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(54) Title: METHODS OF TREATMENT INVOLVING HUMAN MDA-7

(57) Abstract: The present invention relates to gene therapy methods for the treatment of human disease. More specifically, the invention is directed to methods for treating a subject with an angiogenesis-related disease. In one embodiment, an adenoviral expression construct comprising a nucleic acid encoding a human MDA-7 protein under the control of a promoter operable in eukaryotic cells, is administered to said patient with a angiogenesis-related disease. The present invention thus provides for treatment of angiogenesis-related disease by through expression of mda-7 and inhibition angiogenesis. Such diseases include cancer.

## BACKGROUND OF THE INVENTION

This application claims the benefit of priority to U.S. Application Serial No. 60/254,226 filed on December 7, 2000, which is hereby incorporated by reference in its entirety. The government may own rights in the invention pursuant to the specialized Program of Research Excellence (SPORE) in Lung Cancer (P50-CA70907) (J.A. Roth), by Public Health Service grant P01CA78778-01A1, grant number CA73954 from the National Institutes of Health, and grant numbers CA86587 and CA89778 from the National Cancer Institute.

### A. Field of the Invention

The present invention relates generally to the field of gene therapy. More particularly, it concerns a method of administering a therapeutic nucleic acid for the treatment of angiogenesis-related disease by inhibiting angiogensis (anti-angiogenic therapy). In one embodiment, the invention relates to the expression of a nucleic acid encoding human mda-7 protein for the treatment of angiogenesis-related disease by inhibiting angiogenesis.

### B. Description of Related Art

#### 1. Angiogenesis

Blood vessels are constructed by two processes: vasculogenesis, whereby a primitive vascular network is established during embryogenesis from multipotential mesenchymal progenitors; and angiogenesis, in which preexisting vessels send out capillary sprouts to produce new vessels. Endothelial cells are centrally involved in each process. They migrate, proliferate and then assemble into tubes with tight cell-cell connections to contain the blood (Hanahan, 1997). Angiogenesis occurs when enzymes, released by endothelial cells, and leukocytes begin to erode the basement membrane, which surrounds the endothelial cells, allowing the endothelial cells to protrude through the membrane. These endothelial cells then begin to migrate in response to angiogenic stimuli, forming offshoots of the blood vessels, and continue to proliferate until the off-shoots merge with each other to form the new vessels.

angiogenesis, the formation of blood vessels, as described above. Angiogenesis-related diseases may be treated using the methods described in present invention to inhibit endothelial cell proliferation. Angiogenesis-related diseases include, but are not limited to, angiogenesis-dependent cancer, including, for example, solid tumors, blood born tumors such as leukemias, and tumor metastases; benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; rheumatoid arthritis; psoriasis; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, Rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; and wound granulation. The endothelial cell proliferation inhibiting methods of the present invention are useful in the treatment of disease of excessive or abnormal stimulation of endothelial cells. These diseases include, but are not limited to, intestinal adhesions, atherosclerosis. scleroderma, and hypertrophic scars, *i.e.*, keloids. They are also useful in the treatment of diseases that have angiogenesis as a pathologic consequence such as cat scratch disease *(Rochele minalia quintosa)* and ulcers *(Helobacter pylori)*.

3. Cancer

Normal tissue homeostasis is a highly regulated process of cell proliferation and cell death. An imbalance of either cell proliferation or cell death can develop into a cancerous state (Solyanik *et al.*, 1995; Stokke *et al.*, 1997; Mumby and Walter, 1991; Natoli *et al.*, 1998; Magi-Galluzzi *et al.*, 1998). For example, cervical, kidney, lung, pancreatic, colorectal and brain cancer are just a few examples of the many cancers that can result (Erlandsson, 1998; Kolmel, 1998; Mangray and King, 1998; Gertig and Hunter, 1997; Mougin *et al.*, 1998). In fact, the occurrence of cancer is so high that over 500,000 deaths per year are attributed to cancer in the United States alone.

The maintenance of cell proliferation and cell death is at least partially regulated by proto-oncogenes. A proto-oncogene can encode proteins that induce cellular

approaches include the combination of more than one anti-cancer drug, which has proven to increase the response rate of a wide variety of cancers (U.S. Patent 5,824,348; U.S. Patent 5,633,016 and U.S. Patent 5,798,339, incorporated herein by reference). A major side effect of chemotherapy drugs is that they also affect normal tissue cells, with the cells most likely to be affected being those that divide rapidly (*e.g.*, bone marrow, gastrointestinal tract, reproductive system and hair follicles). Other toxic side effects of chemotherapy drugs are sores in the mouth, difficulty swallowing, dry mouth, nausea, diarrhea, vomiting, fatigue, bleeding, hair loss and infection.

Immunotherapy, a rapidly evolving area in cancer research, is yet another option for the treatment of certain types of cancers. For example, the immune system identifies tumor cells as being foreign and thus are targeted for destruction by the immune system. Unfortunately, the response typically is not sufficient to prevent most tumor growths. However, recently there has been a focus in the area of immunotherapy to develop methods that augment or supplement the natural defense mechanism of the immune system. Examples of immunotherapies currently under investigation or in use are immune adjuvants (*e.g.*, *Mycobacterium bovis*, *Plasmodium falciparum*, dinitrochlorobenzene and aromatic compounds) (U.S. Patent 5,801,005; U.S. Patent 5,739,169; Hui and Hashimoto, 1998; Christodoulides *et al.*, 1998), cytokine therapy (*e.g.*, interferons), and (IL-1, GM-CSF and TNF) (Bukowski *et al.*, 1998; Davidson *et al.*, 1998; Hellstrand *et al.*, 1998) gene therapy (*e.g.*, TNF, IL-1, IL-2, p53) (Qin *et al.*, 1998; Austin-Edward and Villaseca, 1998; U.S. Patent 5,830,880 and U.S. Patent 5,846,945) and monoclonal antibodies (*e.g.*, anti-ganglioside GM2, anti-HER-2, anti-p185) (Pietras *et al.*, 1998; Hanibuchi *et al.*, 1998; U.S. Patent 5,824,311).

4. Gene Therapy

Gene therapy is an emerging field in biomedical research with a focus on the treatment of disease by the introduction of therapeutic recombinant nucleic acids into somatic cells of patients. Various clinical trials using gene therapies have been initiated and include the treatment of various cancers, AIDS, cystic fibrosis, adenosine deaminase

was characterized but no function has been attributed yet to this putative cytokine[17]. The rat MDA-7 homolog C49a (Mob-5) is 78% homologous to the mda-7 gene and has been linked to wound healing (Soo *et al.* 1999; Zhang *et al.*, 2000). Mob-5 was also shown to be a secreted protein and a putative cell surface receptor was identified on ras transformed cells (Zhang *et al.*, 2000). Therefore, homologues of the mda-7 gene and the secreted MDA-7 protein are expressed and secreted in various species. However, no data has emerged to show MDA-7 has cytokine activity. Such activity has ramifications for the treatment of a wide variety of diseases and infections by promoting therapeutic immune responses or enhancing immunogenicity of an antigen.

## SUMMARY OF THE INVENTION

It is, therefore, an objective of the present invention to provide a method for treating a patient exhibiting an angiogenesis-related disease comprising administering a therapeutic nucleic acid encoding human MDA-7 protein under the control of a promoter operable in eukaryotic cells, wherein expression of mda-7 inhibits angiogenesis.

In certain embodiments the angiogenesis-related diseases are angiogenesis-dependent cancer, benign tumors, rheumatoid arthritis, psoriasis, ocular angiogenic diseases, Osler-Webber Syndrome, myocardial angiogenesis, plaque neovascularization, telangiectasia, hemophiliac joints, angiofibroma, wound granulation, cat scratch disease, ulcers, intestinal adhesions, atherosclerosis, scleroderma, and/or hypertrophic scars (keloids).

In further embodiments angiogenesis-dependent cancers are further defined as solid tumors, blood born tumors such as leukemias, and/or tumor metastases. In additional embodiments benign tumors are further defined as hemangiomas, acoustic neuromas, neurofibromas, trachomas, and/or pyogenic granulomas. In still further embodiments ocular angiogenic diseases are further defined as diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, and/or Rubeosis.

Preferably, the nucleic acid of the present invention is administered by injection. Other embodiments include the administering of the nucleic acid by multiple injections. In certain embodiments, the injection is performed local, regional or distal to a disease or tumor site. In preferred embodiments, the administering of nucleic acid is via continuous infusion, intratumoral injection, intraperitoneal, or intravenous injection. In certain preferred embodiments, the nucleic acid is administered to the tumor bed prior to or after; or both prior to and after resection of the tumor. In preferred embodiments, the nucleic acid is administered to the patient before, during, or after chemotherapy, biotherapy, immunotherapy, surgery or radiotherapy. Preferably the patient is a human. In other embodiments the patient is a cancer patient.

In preferred embodiments, the nucleic acid encodes amino acids from 49 to 206, 75 to 206, 100 to 206, 125 to 206, 150 to 206, 175 to 206, or 182 to 206 of SEQ ID NO:2. In still further embodiments the nucleic acid encodes or encodes at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, or 206 contiguous amino acids of SEQ ID NO:2.

In certain preferred embodiments, the nucleic acid further comprises nucleotides encoding a secretory signal sequence. In more preferred embodiments, the nucleic acid further comprises secretory signal sequence defined as a positively charged N-terminal region in combination with a hydrophobic core.

Additional embodiments encompass the administration of a chemotherapeutic agent prior, after or before the nucleic acid molecule. In still further embodiments the chemotherapeutic agent is a DNA damaging agent. DNA damaging agent is further defined as gamma-irradiation, X-rays, proton-beam irradiation, UV-irradiation, microwaves, electronic emissions, adriamycin, 5-fluorouracil (5FU), etoposide (VP-16), camptothecin, actinomycin-D, mitomycin C, cisplatin (CDDP), or hydrogen peroxide. While in other embodiments DNA damaging agents are further defined as cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, bisulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen, taxol, transplatinum, 5-fluorouracil, vincristin, vinblastin and methotrexate or any analog or derivative variant thereof.

In certain embodiments the nucleic acid is comprised within a viral vector or in a lipid composition.

Additional embodiments of the invention encompass the use of a purified protein composition comprising MDA-7 protein, truncated versions of MDA-7, and peptides derived from MDA-7 amino acid sequence administered to cells or subjects for the inhibition of angiogenesis.

Other embodiments of the invention concern MDA-7's cytokine activity. The present invention includes methods for promoting an immune response in a subject comprising providing to the subject an effective amount of MDA-7 to promote an immune response. The promotion of an immune response is evidenced by an increase of cytokine expression or activity, proliferation of T cells or a population of T cells (for example, helper, cytotoxic, NK cells) , proliferation of B cells or a population of B cells, cytotoxic T cell activity, or antibody production.

A subject may be given MDA-7 or the antigen more than one time, such as two, three, four times or more. MDA-7 and the antigen may be given at the same time or at different times. Furthermore, it is contemplated that these compounds can be provided to a subject intravenously, directly, intraperitoneally, regionally, systemically, or orally.

It is contemplated that embodiments discussed herein with respect to one method of the invention may be implemented with respect to other methods of the invention.

As used herein the specification, "a" or "an" may mean one or more, unless clearly indicated otherwise. As used herein in the claim(s), when used in conjunction with the word "comprising," the words "a" or "an" may mean one or more than one. As used herein "another" may mean at least a second or more.

## BRIEF DESCRIPTION OF THE FIGURES

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

**FIG. 1.** Schematic illustration of Ad-vectors. Replication-deficient human type 5 Adenovirus (Ad5) carrying the mda-7 (or luciferase gene) linked to an internal CMVIE promoter and followed by SV40 polyadenylation (pA) signal were used. In addition, Ad-CMVp(A) (empty vector ) was used as control.

**FIG 2A.** T47D cells treated with Ad-mda7 at varying MOIs (viral particle/cell). **FIG 2B.** MCF-7 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell).

**FIG. 10.** H460 cells were infected with increasing MOIs of Ad-mda7 or Ad-luc and 48 h later processed for MDA7 surface expression and analyzed by FACS.

**FIG. 11A.** Soluble MDA-7 (sMDA7) kills tumor cells. H1299 cells were challenged with the following samples and percentage dead cells evaluated after 48 hours: 1) Ad-mda7 virus, positive control infected at 1000 Vp/cell; 2) Soluble MDA7 supernatant from H1299 infected cells with Ad-mda-7 (1000 vp/cell); 3) Ad-luc virus, control infected at 1000 Vp/ cell; 4) supernatant from H1299 infected cells with Ad-luc (1000 vp/cell); 5) Ad-p53 virus, positive control infected at 20 Vp/cell; 6) a separate stock of soluble MDA-7 supernatant obtained from 293 cells infected with Ad-mda-7 (sup M, 500 Vp/cell); and 7) a separate stock of soluble MDA-7 supernatant obtained from modified serum-free 293 cells infected with Ad-mda-7 (sup P, 500 Vp/cell). All the supernatants used in this experiment were filtered through a 0.1 micron filter prior to challenge with H1299 cells. **FIG. 11B.** H1299 cells were challenged with soluble MDA-7 supernatant from four different stocks and percentage dead cells evaluated after 48 hours: 1) 293*NF: Non-filtered supernatant obtained from modified 293 cells (cells were grown in serum-free conditions); 2) 293*F: 0.1 micron filtered supernatant obtained from modified 293 cells; 3) 293F: 0.1 micron filtered supernatant obtained from regular 293 cells (FBS +); and 4) H1299F: 0.1 micron filtered supernatant obtained from H1299 cells. D0 is non-diluted material whereas D1:1; D1:5, D1:10 indicate the dilutions used. Control undiluted supernatant from Ad-luc treated H1299 cells demonstrated 20% dead cells.

**FIG. 12.** Combination with Tamoxifen. Ad-mda7 has been combined with tamoxifen and evaluated for anti-tumor effects in breast cancer cell lines. The graphs demonstrate that combining these two agents provides superior anti-tumor activity compared to either agent alone.

**FIG. 13.** Combination with Adriamycin. Ad-mda7 has been combined with adriamycin and evaluated for anti-tumor effects in breast cancer cell lines. The graphs

significance of tumor volume changes were calculated using the student's *t*- test. Each time point represents the mean tumor volume for each group. Bars represent standard error.

**FIG. 18.** *mda-7* gene expression and apoptotic cell death following Ad-*mda7* treatment *in vivo*. Subcutaneous H1299 tumors from animals receiving *Ad-luc* or Ad-*mda7* were harvested 48 hours after treatment. Quantitative analysis of tumor tissues demonstrated 15% of tumor cells treated with Ad-mda7 expressing MDA-7 (a) and 17% of tumor cells undergoing apoptosis (b).

**FIG. 19.** Downregulation of CD31 and upregulation of TRAIL expression by *mda-7*. Lower CD31 expression was observed in tumors treated with Ad-*mda7* (9%) than in tumors receiving no treatment (40%) or Ad-*luc* (28%) (a). Expression of TRAIL was higher in tumors treated with Ad-*mda7* (20%) than in tumors receiving no treatment (1%) or Ad-*luc* (4%) (b).

**FIG. 20.** Summary of immunihistochemistry analyses of patients treated intratumorally with Ad-mda7. "Pt" indicates patient. Time indicates the number of hours after injection that tumors were resected for immunohistochemistry. References to MDA-7 indicate positive expression for MDA-7 protein at center of tumor (at injection site) or at the periphery (>1 cm from injection site). TUNEL data is presented as well. Ad-mda7 injection into tumors in humans results in high levels of mda-7 transgene expression and high levels levels of apoptosis induction.

**FIG. 21.** Summary of DNA PCR data demonstrating high levels of Ad-mda7 DNA in the center of injected lesions in patients. Tumors were injected with Ad-mda7 and at the time indictated, were resected. Approximately 2 mm sections were obtained from the center of each tumor (corresponding to the injection site) and were subjected to quantitative DNA-PCR analysis. Data are plotted as numbers of Ad-mda7 DNA copies

As mentioned above, tumor suppressors play an important role in cancer biology. One of these, p53 tumor suppressor proto-oncogene is essential for the maintenance of the non-tumorogenic phenotype of cells (reviewed by Soddu and Sacchi, 1998). Approximately 50% of all cancers have been found to be associated with mutations of the p53 gene, which result in the loss of p53 tumor suppressor properties (Levine *et al.*, 1991; Vogelstein and Kinzler, 1992; Hartmann *et al.*, 1996a; Hartmann *et al.*, 1996b). Mutations in the p53 gene also result in the prolongation of the p53 half-life in cells and the overexpression of p53 protein. In normal cells, p53 is undetectable due to its high turnover rate. The high incidence of cancer related to mutations of the p53 gene has prompted many research groups to investigate p53 as a route of cancer treatment via gene replacement.

MDA-7, another putative tumor suppressor, has been shown to suppress the growth of cancer cells that are p53-wild-type, p53-null and p53-mutant. Also, the upregulation of the apoptosis-related Bax gene in p53 null cells indicates that MDA-7 is capable of using p53-independent mechanisms to induce the destruction of cancer cells. These characteristics suggest that MDA-7 has broad therapeutic potential as an inducer of apoptosis.

The present invention contemplates the treatment of a patient in need of anti-angiogenesis therapy, including cancer, by identifying patients with such diseases and expressing human mda-7 polypeptide by means of nucleic acid transfer. The treatment of such an angiogenesis-related disease in one embodiment involves the intratumoral administration of either human mda-7 expression construct to endothelial cells of the disease related vascular bed. The endothelial cells then express human MDA-7, resulting in the inhibition of angiogenesis.

## A. MDA-7

The cDNA encoding the MDA-7 protein (also referred to mda-7 herein) has been described by Jiang *et al.*, 1995 (WO 95/11986, incorporated herein by reference), where

Jiang *et al.* (1996) report findings that mda-7 is a potent growth suppressing gene in cancer cells of diverse origins including breast, central nervous system, cervix, colon, prostate, and connective tissue. A colony inhibition assay was used to demonstrate that elevated expression of MDA-7 enhanced growth inhibition in human cervical carcinoma (HeLa), human breast carcinoma (MCF-7 and T47D), colon carcinoma (LS174T and SW480), nasopharyngeal carcinoma (HONE-1), prostate carcinoma (DU-145), melanoma (HO-1 and C8161), glioblastome multiforme (GBM-18 and T98G), and osteosarcoma (Saos-2). Mda-7 overexpression in normal cells (HMECs, HBL-100, and CREF-Trans6) showed limited growth inhibition indicating that mda-7 transgene effects are not manifest in normal cells. In summary, growth inhibition by elevated expression of MDA-7 is more effective *in vitro* in cancer cells than in normal cells.

Su *et al.* (1998) reported investigations into the mechanism by which MDA-7 suppressed cancer cell growth. The studies reported that ectopic expression of MDA-7 in breast cancer cell lines MCF-7 and T47D induced apoptosis as detected by cell cycle analysis and TUNEL assay without an effect on the normal HBL-100 cells. Western blot analysis of cell lysates from cells infected with adenovirus mda-7 ("Ad-mda-7") showed an upregulation of the apoptosis stimulating protein BAX. Ad-mda-7 infection elevated levels of BAX protein only in MCF-7 and T47D cells and not normal HBL-100 or HMEC cells. These data lead the investigators to evaluate the effect of *ex vivo* Ad-mda-7 transduction on xenograft tumor formation of MCF-7 tumor cells. *Ex vivo* transduction resulted in the inhibition of tumor formation and progression in the tumor xenograft model. The mechanisms by which this novel tumor suppressor molecule acts are beginning to be investigated.

**B. Angiogenesis-Related Disease and Mda-7**

The methods of the present invention are useful for treating endothelial cell-related diseases and disorders. A particularly important endothelial cell process is angiogenesis, the formation of blood vessels, as described above. Angiogenesis-related

granulation. The endothelial cell proliferation inhibiting methods of the present invention are useful in the treatment of disease of excessive or abnormal stimulation of endothelial cells. These diseases include, but are not limited to, intestinal adhesions, atherosclerosis. scleroderma, and hypertrophic scars, *i.e.*, keloids. They are also useful in the treatment of diseases that have angiogenesis as a pathologic consequence such as cat scratch disease *(Rochele minalia quintosa)* and ulcers *(Helobacter pylori)*.

Cancer has become one of the leading causes of death in the Western world, second only behind heart disease. Current estimates project that one person in three in the U.S. will develop cancer, and that one person in five will die from cancer. Cancers can be viewed as altered cells that have lost the normal growth-regulating mechanisms.

There currently are three major categories of oncogenes, reflecting their different activities. One category of oncogenes encodes proteins that induce cellular proliferation. A second category of oncogenes, called tumor-suppressors genes or anti-oncogenes, function to inhibit excessive cellular proliferation. The third category of oncogenes, either block or induce apoptosis by encoding proteins that regulate programmed cell death.

The cDNA encoding the mda-7 protein has been described by Jiang *et al.*, 1995 (WO 9511986), where the protein encoded by the mda-7 cDNA was recognized as a potential regulator of melanoma progression. Jiang *et al.* used a subtractive hybridization technique (Jiang and Fisher, 1993) to identify genes involved in the regulation of growth and differentiation in human melanoma cells. A cDNA library prepared by subtraction hybridization of cDNAs prepared form actively proliferating human HO-1 melanoma cells against cDNAs prepared from interferon (IFN-β) and mezerin differentiated human HO-1 melanoma cells was used to identify several melanoma differentiation associated (mda) cDNAs. The cDNA for mda-7 was identified as having elevated expression levels in the differentiated melanoma cells.

the investigators to evaluate the effect of *ex vivo* Ad-mda-7 transduction on xenograft tumor formation of MCF-7 tumor cells. *Ex vivo* transduction resulted in the inhibition of tumor formation and progression in the tumor xenograft model. Mda-7 has been shown to be effective in tumor cell specific apoptotic induction. Thus, one embodiment of the present invention is the treatment of various angiogenesis-related diseases with a mda-7 adenoviral construct.

PCT publication number WO 98/28425 describes a cytokine molecule allegedly related to IL-10. This molecule, designated IL-BKW, appears to be derived from the same gene as mda-7. The mature form of IL-BKW was said to begin at about residue 47 or 49 of the mda-7 coding region, and continue some 158-160 residues, *i.e.*, to residues 206 of the mda-7 sequence. Thus, a preferred molecule would preferably lack all or part of both the putative signal sequence (residues 1-25) and a putative membrane spanning hydrophobic domain (residues 26-45) of full length mda-7.

Truncated molecules of mda-7 are also contemplated. For example, while molecules beginning approximately at mda-7 amino acid residues 46-49 are the largest molecules, further N-terminal truncations are within the scope of the invention. Thus, specifically contemplated are molecules start at residue 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, and 182, and terminate at residue 206. In additional embodiments, residues 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, and 46 are included with other contiguous residues of MDA-7, as shown in SEQ ID NO:2.

inflammatory bowel disease, osteoarthritis, leiomyomas, ademonas, lipomas, hemangiomas, fibromas, vascular occlusion, restenosis, atherosclerosis, pre-neoplastic lesions, carcinoma *in situ*, oral hairy leukoplakia or psoriasis may be the subject of treatment.

## C. Cytokines and Immune Stimulation

Cytokines can promote an immune response to a compound. Because MDA-7 has cytokine activity, this effect can be utilized for therapeutic and preventative methods. It is contemplated that an immune response against any of the antigens described below would effect a therapeutic effect against a disease or condition associated with the antigen or effect a preventative therapy against that disease or condition.

In some embodiments, MDA-7 can be used to promote or enhance an immune response against an antigen associated with a disease or condition. In some embodiments of the invention, antigens may be associated or derived from microbial, fungal, viral, or tumor agents. Examples of microbes from which antigens of the invention may be drawn include, but are not limited to, the 83 or more distinct serotypes of pneumococci, streptococci such as *S. pyogenes, S. agalactiae, S. equi, S. canis, S. bovis, S. equinus, S. anginosus, S. sanguis, S. salivarius, S. mitis, S. mutans*, other viridans streptococci, peptostreptococci, other related species of streptococci, enterococci such as *Enterococcus faecalis, Enterococcus faecium*, Staphylococci, such as *Staphylococcus epidermidis, Staphylococcus aureus*, particularly in the nasopharynx, *Hemophilus influenzae*, pseudomonas species such as *Pseudomonas aeruginosa, Pseudomonas pseudomallei, Pseudomonas mallei*, brucellas such as *Brucella melitensis, Brucella suis, Brucella abortus, Bordetella pertussis, Neisseria meningitidis, Neisseria gonorrhoeae, Moraxella catarrhalis, Corynebacterium diphtheriae, Corynebacterium ulcerans, Corynebacterium pseudotuberculosis, Corynebacterium pseudodiphtheriticum, Corynebacterium urealyticum, Corynebacterium hemolyticum, Corynebacterium equi*, etc. *Listeria monocytogenes, Nocordia asteroides*, Bacteroides species, Actinomycetes species, *Treponema pallidum*, Leptospirosa species and related organisms. The invention may

Furthermore, it is contemplated that all or part of MDA-7 may be part of a fusion protein with another cytokine molecule and/or with an antigen against which an immune response is desired. This could be administered to a subject to induce or promote an immune response against the antigen.

MDA-7 can also be administered to a patient in combination with a tumoricidal compound or a compound with a tumor cytostatic effect to enhance the ability of that compound to inhibit or kill tumor cells. Such compounds include tumor suppressors and compounds discussed below under the heading "Combination Therapy." In some embodiments, the tumoricidal compound is p53, Rb, WT, FHIT, p16, PTEN, APC, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, zac1, p73, VHL, MMAC1, DBCCR-1, FCC, rsk-3, p27, or TRAIL.

An immune response against tumor antigens can also be implemented with MDA-7. Tumor antigens include PSA, CEA, MART, MAGE1, MAGE 3, gp100, BAGE, GAGE, TRP-1, TRP-2, or PMSA. Uses for inducing a response against tumor antigens are specifically contemplated and can be found in U.S. Patents 5,552,293 and 6,132,980, which are specifically incorporated by reference.

A number of assays are well known to those of skill in the art regarding assaying for induction, promotion, or enhancement of an immune response, some of which are described in an example below and in the references incorporated by reference herein. One assay involves detecting an increase of expression of other cytokines, such as IL-6, TNF, IFN, GM-CSF, CSF, or other IL cytokines. Such cytokines may be administered to a subject in combination with the MDA-7 compositions described herein and any other composition described herein. It is contemplated that any embodiment discussed with respect to MDA-7 and inhibition of angiogenesis or treatment of cancer may be applied to methods of promoting an immune response.

represents coding regions of a polypeptide and eliminates introns and other regulatory regions.

It also is contemplated that a given MDA-7-encoding nucleic acid or *mda-7* gene from a given cell may be represented by natural variants or strains that have slightly different nucleic acid sequences but, nonetheless, encode a MDA-7 polypeptide; a human MDA-7 polypeptide is a preferred embodiment. Consequently, the present invention also encompasses derivatives of MDA-7 with minimal amino acid changes, but that possess the same activity.

The term "gene" is used for simplicity to refer to a functional protein, polypeptide, or peptide-encoding unit. As will be understood by those in the art, this functional term includes genomic sequences, cDNA sequences, and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides, domains, peptides, fusion proteins, and mutants. The nucleic acid molecule encoding MDA-7 or another therapeutic polypeptide may comprise a contiguous nucleic acid sequence of the following lengths or at least the following lengths: 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 441, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800, 810, 820, 830, 840, 850, 860,

The term "biologically functional equivalent" is well understood in the art and is further defined in detail herein. Accordingly, sequences that have about 70%, about 71%, about 72%, about 73%, about 74%, about 75%, about 76%, about 77%, about 78%, about 79%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99%, and any range derivable therein, such as, for example, about 70% to about 80%, and more preferably about 81% and about 90%; or even more preferably, between about 91% and about 99%; of amino acids that are identical or functionally equivalent to the amino acids of SEQ ID NO:2 will be sequences that are "essentially as set forth in SEQ ID NO:2" provided the biological activity of the protein is maintained. In particular embodiments, the biological activity of a MDA-7 protein, polypeptide or peptide, or a biologically functional equivalent, comprises inhibiting angiogenesis, inhibiting or killing cancer cells, inducing apoptosis, and/or inducing an immune response. In certain other embodiments, the invention concerns isolated DNA segments and recombinant vectors that include within their sequence a nucleic acid sequence essentially as set forth in SEQ ID NO:1. The term "essentially as set forth in SEQ ID NO:1" is used in the same sense as described above and means that the nucleic acid sequence substantially corresponds to a portion of SEQ ID NO:1 and has relatively few codons that are not identical, or functionally equivalent, to the codons of SEQ ID NO:1. Again, DNA segments that encode proteins, polypeptide or peptides exhibiting MDA-7 activity will be most preferred.

In particular embodiments, the invention concerns isolated nucleic acid segments and recombinant vectors incorporating DNA sequences that encode MDA-7 polypeptides or peptides that include within its amino acid sequence a contiguous amino acid sequence in accordance with, or essentially corresponding to MDA-7 polypeptides.

Vectors of the present invention are designed, primarily, to transform endothelial cells with a therapeutic mda-7 gene under the control of regulated eukaryotic promoters

| Element | Inducer | References |
|---|---|---|
| Proliferin | Phorbol Ester-TPA | Mordacq and Linzer, 1989 |
| Tumor Necrosis Factor | PMA | Hensel *et al.*, 1989 |
| Thyroid Stimulating Hormone α Gene | Thyroid Hormone | Chatterjee *et al.*, 1989 |

| Promoter/Enhancer | References |
|---|---|
| Insulin | Edlund *et al.*, 1985 |
| Neural Cell Adhesion Molecule (NCAM) | Hirsch *et al.*, 1990 |
| $a_{1\text{-Antitrypain}}$ | Latimer *et al.*, 1990 |
| H2B (TH2B) Histone | Hwang *et al.*, 1990 |
| Mouse or Type I Collagen | Rippe *et al.*, 1989 |
| Glucose-Regulated Proteins (GRP94 and GRP78) | Chang *et al.*, 1989 |
| Rat Growth Hormone | Larsen *et al.*, 1986 |
| Human Serum Amyloid A (SAA) | Edbrooke *et al.*, 1989 |
| Troponin I (TN I) | Yutzey *et al.*, 1989 |
| Platelet-Derived Growth Factor | Pech *et al.*, 1989 |
| Duchenne Muscular Dystrophy | Klamut *et al.*, 1990 |
| SV40 | Banerji *et al.*, 1981; Moreau *et al.*, 1981; Sleigh and Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek *et al.*, 1987; Kuhl *et al.*, 1987 Schaffner *et al.*, 1988 |
| Polyoma | Swartzendruber and Lehman, 1975; Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Tyndell *et al.*, 1981; Dandolo *et al.*, 1983; Hen *et al.*, 1986; Sakai *et al.*, 1988; Campbell and Villarreal, 1988 |
| Retroviruses | Kriegler and Botchan, 1983; Kriegler *et al.*, 1984a,b; Bosze *et al.*, 1986; Miksicek *et al.*, 1986; Celander and Haseltine, 1987; Thiesen *et al.*, 1988; Celander *et al.*, 1988; Chol *et al.*, 1996; Reisman and Rotter, 1989 |
| Papilloma Virus | Campo *et al.*, 1983; Lusky *et al.*, 1983; Spandidos and Wilkie, 1983; Spalholz *et al.*, 1985; Lusky and Botchan, 1986; Cripe *et al.*, 1987; Gloss *et al.*, 1987; Hirochika *et al.*, 1987, Stephens and Hentschel, 1987 |
| Hepatitis B Virus | Bulla and Siddiqui, 1988; Jameel and Siddiqui, 1986; Shaul and Ben-Levy, 1987; Spandau and Lee, 1988 |
| Human Immunodeficiency Virus | Muesing *et al.*, 1987; Hauber and Cullan, 1988; Jakobovits *et al.*, 1988; Feng and Holland, 1988; Takebe |

number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between elements is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Aside from this operational distinction, enhancers and promoters are very similar entities.

Promoters and enhancers have the same general function of activating transcription in the cell. They are often overlapping and contiguous, often seeming to have a very similar modular organization. Taken together, these considerations suggest that enhancers and promoters are homologous entities and that the transcriptional activator proteins bound to these sequences may interact with the cellular transcriptional machinery in fundamentally the same way.

Preferred for use in the present invention is the cytomegalovirus (CMV) promoter. This promoter is commercially available from Invitrogen in the vector pcDNAIII, which

## E. Gene Transfer

### 1. Viral Transformation

#### a. Adenoviral Infection

One method for delivery of the recombinant DNA involves the use of an adenovirus expression vector. Although adenovirus vectors are known to have a low capacity for integration into genomic DNA, this feature is counterbalanced by the high efficiency of gene transfer afforded by these vectors. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to ultimately express a recombinant gene construct that has been cloned therein.

The vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization or adenovirus, a 36 kb, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kb (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target-cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are cis elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant

Racher *et al.* (1995) have disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 h. The medium is then replaced with 50 ml of fresh medium and shaking initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking commenced for another 72 h.

The adenovirus vector may be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the transforming construct at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical to the invention. The polynucleotide encoding the gene of interest may also be inserted in lieu of the deleted E3 region in E3

sequence found upstream from the gag gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a gene of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and packaging components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

Concern with the use of defective retrovirus vectors is the potential appearance of wild-type replication-competent virus in the packaging cells. This can result from recombination events in which the intact sequence from the recombinant virus inserts upstream from the gag, pol, env sequence integrated in the host cell genome. However, packaging cell lines are available that should greatly decrease the likelihood of recombination (Markowitz *et al.*, 1988; Hersdorffer *et al.*, 1990).

c. AAV Infection

Adeno-associated virus (AAV) is an attractive vector system for use in the present invention as it has a high frequency of integration and it can infect nondividing cells, thus making it useful for delivery of genes into mammalian cells in tissue culture (Muzyczka,

*et al.*, 1988; Samulski *et al.*, 1989; each incorporated herein by reference) and an expression plasmid containing the wild-type AAV coding sequences without the terminal repeats, for example pIM45 (McCarty *et al.*, 1991; incorporated herein by reference). The cells are also infected or transfected with adenovirus or plasmids carrying the adenovirus genes required for AAV helper function. rAAV virus stocks made in such fashion are contaminated with adenovirus which must be physically separated from the rAAV particles (for example, by cesium chloride density centrifugation). Alternatively, adenovirus vectors containing the AAV coding regions or cell lines containing the AAV coding regions and some or all of the adenovirus helper genes could be used (Yang *et al.*, 1994a; Clark *et al.*, 1995). Cell lines carrying the rAAV DNA as an integrated provirus can also be used (Flotte *et al.*, 1995).

d. Other Viral Vectors

Other viral vectors may be employed as constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988) and herpesviruses may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

A molecularly cloned strain of Venezuelan equine encephalitis (VEE) virus has been genetically refined as a replication competent vaccine vector for the expression of heterologous viral proteins (Davis *et al.*, 1996). Studies have demonstrated that VEE infection stimulates potent CTL responses and has been sugested that VEE may be an extremely useful vector for immunizations (Caley *et al.*, 1997). It is contemplated in the present invention, that VEE virus may be useful in targeting dendritic cells.

With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. In vitro studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich *et al.*,

a. Electroporation

In certain preferred embodiments of the present invention, the gene construct is introduced into the dendritic cells via electroporation. Electroporation involves the exposure of a suspension of cells and DNA to a high-voltage electric discharge.

Transfection of eukaryotic cells using electroporation has been quite successful. Mouse pre-B lymphocytes have been transfected with human kappa-immunoglobulin genes (Potter *et al.*, 1984), and rat hepatocytes have been transfected with the chloramphenicol acetyltransferase gene (Tur-Kaspa *et al.*, 1986) in this manner.

It is contemplated that electroporation conditions for endothelial cells from different sources may be optimized. One may particularly wish to optimize such parameters as the voltage, the capacitance, the time and the electroporation media composition. The execution of other routine adjustments will be known to those of skill in the art.

b. Particle Bombardment

Another embodiment of the invention for transferring a naked DNA construct into cells involves particle bombardment. This method depends on the ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). The microprojectiles used have consisted of biologically inert substances such as tungsten, platinum or gold beads.

It is contemplated that in some instances DNA precipitation onto metal particles would not be necessary for DNA delivery to a recipient cell using particle bombardment. It is contemplated that particles may contain DNA rather than be coated with DNA. Hence it is proposed that DNA-coated particles may increase the level of DNA delivery via particle bombardment but are not, in and of themselves, necessary.

Accordingly, it is contemplated that one may wish to adjust various of the bombardment parameters in small scale studies to fully optimize the conditions. One may particularly wish to adjust physical parameters such as gap distance, flight distance, tissue distance and helium pressure. One also may optimize the trauma reduction factors by modifying conditions which influence the physiological state of the recipient cells and which may therefore influence transformation and integration efficiencies. For example, the osmotic state, tissue hydration and the subculture stage or cell cycle of the recipient cells may be adjusted for optimum transformation. The execution of other routine adjustments will be known to those of skill in the art.

c. Calcium Phosphate Co-Precipitation or DEAE-Dextran Treatment

In other embodiments of the present invention, the transgenic construct is introduced to the cells using calcium phosphate co-precipitation. Mouse primordial germ cells have been transfected with the SV40 large T antigen, with excellent results (Watanabe *et al.*, 1997). Human KB cells have been transfected with adenovirus 5 DNA (Graham and Van Der Eb, 1973) using this technique. Also in this manner, mouse L(A9), mouse C127, CHO, CV-1, BHK, NIH3T3 and HeLa cells were transfected with a neomycin marker gene (Chen and Okayama, 1987), and rat hepatocytes were transfected with a variety of marker genes (Rippe *et al.*, 1990).

In another embodiment, the expression construct is delivered into the cell using DEAE-dextran followed by polyethylene glycol. In this manner, reporter plasmids were introduced into mouse myeloma and erythroleukemia cells (Gopal, 1985).

d. Direct Microinjection or Sonication Loading

Further embodiments of the present invention include the introduction of the nucleic acid construct by direct microinjection or sonication loading. Direct microinjection has been used to introduce nucleic acid constructs into Xenopus oocytes (Harland and Weintraub, 1985), and LTK- fibroblasts have been transfected with the thymidine kinase gene by sonication loading (Fechheimer *et al.*, 1987).

interaction of lipid vehicles and plasma proteins is responsible for the disparity between the efficiency of in vitro (Felgner *et al.*, 1987) and *in vivo* gene transfer (Zhu *et al.*, 1993; Philip *et al.*, 1993; Solodin *et al.*, 1995; Liu *et al.*, 1995; Thierry *et al.*, 1995; Tsukamoto *et al.*, 1995; Aksentijevich *et al.*, 1996).

Recent advances in lipid formulations have improved the efficiency of gene transfer *in vivo* (Smyth-Templeton *et al.*, 1997; WO 98/07408). A novel lipid formulation composed of an equimolar ratio of 1,2-bis(oleoyloxy)-3-(trimethyl ammonio)propane (DOTAP) and cholesterol significantly enhances systemic *in vivo* gene transfer, approximately 150-fold. The DOTAP:cholesterol lipid formulation is said to form a unique structure termed a "sandwich liposome". This formulation is reported to "sandwich" DNA between an invaginated bi-layer or 'vase' structure. Beneficial characteristics of these lipid structures include a positive colloidal stabilization by cholesterol, two dimensional DNA packing and increased serum stability.

The production of lipid formulations often is accomplished by sonication or serial extrusion of liposomal mixtures after (I) reverse phase evaporation (II) dehydration-rehydration (III) detergent dialysis and (IV) thin film hydration. Once manufactured, lipid structures can be used to encapsulate compounds that are toxic (chemotherapeutics) or labile (nucleic acids) when in circulation. Lipid encapsulation has resulted in a lower toxicity and a longer serum half-life for such compounds (Gabizon *et al.*, 1990). Numerous disease treatments are using lipid based gene transfer strategies to enhance conventional or establish novel therapies, in particular therapies for treating angiogenesis-related diseasess.

In certain embodiments of the invention, the lipid vehicle may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of lipid-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the lipid vehicle may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further

endothelial cell with the therapeutic expression construct. The routes of administration will vary, naturally, with the location and nature of the lesion, and include, *e.g.*, intradermal, parenteral, intravenous, intramuscular, intranasal, and oral administration and formulation.

Intratumoral injection, or injection into the tumor vasculature is specifically contemplated for discrete, solid, accessible tumors. Local, regional or systemic administration also may be appropriate. For tumors of >4 cm, the volume to be administered will be about 4-10 ml (preferably 10 ml), while for tumors of <4 cm, a volume of about 1-3 ml will be used (preferably 3 ml). Multiple injections delivered as single dose comprise about 0.1 to about 0.5 ml volumes. The viral particles may advantageously be contacted by administering multiple injections to the tumor, spaced at approximately 1 cm intervals.

In the case of surgical intervention, the present invention may be used preoperatively, to render an inoperable tumor subject to resection. Alternatively, the present invention may be used at the time of surgery, and/or thereafter, to treat residual or metastatic disease. For example, a resected tumor bed may be injected or perfused with a formulation comprising mda-7 or an mda-7-encoding construct. The perfusion may be continued post-resection, for example, by leaving a catheter implanted at the site of the surgery. Periodic post-surgical treatment also is envisioned.

Continuous administration also may be applied where appropriate, for example, where a tumor is excised and the tumor bed is treated to eliminate residual, microscopic disease. Delivery via syringe or catherization is preferred. Such continuous perfusion may take place for a period from about 1-2 hours, to about 2-6 hours, to about 6-12 hours, to about 12-24 hours, to about 1-2 days, to about 1-2 wk or longer following the initiation of treatment. Generally, the dose of the therapeutic composition via continuous perfusion will be equivalent to that given by a single or multiple injections, adjusted over a period of time during which the perfusion occurs.

Protein may be administered to a patient in doses of or of at least 0.01. 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0. 9.0, 10, 15, 20, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000 or more ng/ml.

2. Injectable Compositions and Formulations

The preferred method for the delivery of an expression construct encoding human mda-7 protein to endothelial cells in the present invention is via intratumoral injection. However, the pharmaceutical compositions disclosed herein may alternatively be administered parenterally, intravenously, intradermally, intramuscularly, or even intraperitoneally as described in U.S. Patent 5,543,158; U.S. Patent 5,641,515 and U.S. Patent 5,399,363 (each specifically incorporated herein by reference in its entirety).

Injection of nucleic acid constructs may be delivered by syringe or any other method used for injection of a solution, as long as the expression construct can pass through the particular gauge of needle required for injection. A novel needeless injection system has recently been described (U.S. Patent 5,846,233) having a nozzle defining an ampule chamber for holding the solution and an energy device for pushing the solution out of the nozzle to the site of delivery. A syringe system has also been described for use in gene therapy that permits multiple injections of predetermined quantities of a solution precisely at any depth (U.S. Patent 5,846,225).

Solutions of the active compounds as free base or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation

preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The compositions disclosed herein may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its

chemo- or radiotherapeutic intervention, in addition to other pro-apoptotic or cell cycle regulating agents.

Alternatively, the gene therapy may precede or follow the other agent treatment by intervals ranging from minutes to weeks. In embodiments where the other agent and expression construct are applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent and expression construct would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one may contact the cell with both modalities within about 12-24 h of each other and, more preferably, within about 6-12 h of each other. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several d (2, 3, 4, 5, 6 or 7) to several wk (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

Various combinations may be employed, gene therapy is "A" and the secondary agent, such as radio- or chemotherapy, is "B":

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| A/B/A | B/A/B | B/B/A | A/A/B | A/B/B | B/A/A | A/B/B/B | B/A/B/B |
| B/B/B/A | B/B/A/B | A/A/B/B | A/B/A/B | A/B/B/A | B/B/A/A | | |
| B/A/B/A | B/A/A/B | A/A/A/B | B/A/A/A | A/B/A/A | A/A/B/A | | |

Administration of the therapeutic expression constructs of the present invention to a patient will follow general protocols for the administration of chemotherapeutics, taking into account the toxicity, if any, of the vector. It is expected that the treatment cycles would be repeated as necessary. It also is contemplated that various standard therapies, as well as surgical intervention, may be applied in combination with the described endothelial cell therapy.

same range and therefore deliver a maximum dose at the same depth, and (3) protons being relatively heavy do not deviate much from a straight line as they come to rest.

To realize the full potential of the proton beam in the treatment of cancer and other diseases responsive to radiation treatment, it is necessary for the physician to known the exact location of the site to be treated and the characteristics of the tissue overlying the treatment site. It is only with advent of new imaging techniques such as computed tomography (CT scanning) and magnetic resonance imaging (MRI) that such information is now available with the required accuracy. Proton therapy for the treatment of cancer patients is now feasible.

The terms "contacted" and "exposed," when applied to a cell, are used herein to describe the process by which a therapeutic construct and a chemotherapeutic or radiotherapeutic agent are delivered to a target cell or are placed in direct juxtaposition with the target cell. To achieve cell killing or stasis, both agents are delivered to a cell in a combined amount effective to kill the cell or prevent it from dividing.

3. Genes

In yet another embodiment, the secondary treatment is a secondary gene therapy. Delivery of a vector encoding MDA-7 in conjuction with a second vector encoding one of the following gene products will have a combined anti-hyperproliferative effect on target tissues. Alternatively, a single vector encoding both genes may be used.

a. Inducers of Cellular Proliferation

The proteins that induce cellular proliferation further fall into various categories dependent on function. The commonality of all of these proteins is their ability to regulate cellular proliferation. For example, a form of PDGF, the sis oncogene is a secreted growth factor. Oncogenes rarely arise from genes encoding growth factors, and at the present, sis is the only known naturally occurring oncogenic growth factor. In one embodiment of the present invention, it is contemplated that anti-sense mRNA directed to

It is mutated in over 50% of human NSCLC (Hollstein et al., 1991) and in a wide spectrum of other tumors.

The p53 gene encodes a 393-amino acid phosphoprotein that can form complexes with host proteins such as large-T antigen and E1B. The protein is found in normal tissues and cells, but at concentrations which are minute by comparison with transformed cells or tumor tissue

Wild-type p53 is recognized as an important growth regulator in many cell types. Missense mutations are common for the p53 gene and are essential for the transforming ability of the oncogene. A single genetic change prompted by point mutations can create carcinogenic p53. Unlike other oncogenes, however, p53 point mutations are known to occur in at least 30 distinct codons, often creating dominant alleles that produce shifts in cell phenotype without a reduction to homozygosity. Additionally, many of these dominant negative alleles appear to be tolerated in the organism and passed on in the germ line. Various mutant alleles appear to range from minimally dysfunctional to strongly penetrant, dominant negative alleles (Weinberg, 1991).

Another inhibitor of cellular proliferation is p16. The major transitions of the eukaryotic cell cycle are triggered by cyclin-dependent kinases, or CDK's. One CDK, cyclin-dependent kinase 4 (CDK4), regulates progression through the G1. The activity of this enzyme may be to phosphorylate Rb at late G1. The activity of CDK4 is controlled by an activating subunit, D-type cyclin, and by an inhibitory subunit, the p16INK4 has been biochemically characterized as a protein that specifically binds to and inhibits CDK4, and thus may regulate Rb phosphorylation (Serrano *et al.*, 1993; Serrano *et al.*, 1995). Since the p16INK4 protein is a CDK4 inhibitor (Serrano, 1993), deletion of this gene may increase the activity of CDK4, resulting in hyperphosphorylation of the Rb protein. p16 also is known to regulate the function of CDK6.

Subsequent to its discovery, it was shown that Bcl-2 acts to suppress cell death triggered by a variety of stimuli. Also, it now is apparent that there is a family of Bcl-2 cell death regulatory proteins which share in common structural and sequence homologies. These different family members have been shown to either possess similar functions to Bcl-2 (*e.g.*, BclXL, BclW, Mcl-1, A1, Bfl-1) or counteract Bcl-2 function and promote cell death (*e.g.*, Bax, Bak, Bik, Bim, Bid, Bad, Harakiri).

4. Other agents

It is contemplated that other agents may be used in combination with the present invention to improve the therapeutic efficacy of treatment. These additional agents include immunomodulatory agents, agents that affect the upregulation of cell surface receptors and GAP junctions, cytostatic and differentiation agents, inhibitors of cell adehesion, or agents that increase the sensitivity of the endothelial cells to apoptotic inducers. Immunomodulatory agents include tumor necrosis factor; interferon alpha, beta, and gamma; IL-2 and other cytokines; F42K and other cytokine analogs; or MIP-1, MIP-1beta, MCP-1, RANTES, and other chemokines. It is further contemplated that the upregulation of cell surface receptors or their ligands such as Fas/Fas ligand, DR4 or DR5/TRAIL would potentiate the apoptotic inducing abililties of the present invention by establishment of an autocrine or paracrine effect on endothelial cells. Increases intercellular signaling by elevating the number of GAP junctions would increase the anti-hyperproliferative effects on the neighboring endothelial cell population. In other embodiments, cytostatic or differentiation agents can be used in combination with the present invention to improve the anti-hyerproliferative efficacy of the treatments. Inhibitors of cell adehesion are contemplated to improve the efficacy of the present invention. Examples of cell adhesion inhibitors are focal adhesion kinase (FAKs) inhibitors and Lovastatin. It is furhter contemplated that other agents that increase the sensitivity of a endothelial cell to apoptosis, such as the antibody c225, could be used in combination with the present invention to improve the treatment efficacy.

d. *In vitro* transfection

Cells were plated at a density of 5x105 cells per 60 mm2 in RPMI/10% FBS media and grown in 5% CO2 at 37°C.

e. Recombinant Adenovirus Production

Replication deficient human type 5 Adenovirus (Ad5) carrying the nucleic acid encoding extracellular human mda-7 (or Luciferase gene) linked to an internal CMVIE promoter and followed by SV40 polyadenylation (pA) signal were constructed. A third control vector with just the CMV-pA construct also was constructed. The Ad-5 vectors harboring the gene cassettes were co-transfected with plasmid pJM17 (Graham and Prevec 1992) in 293 cells to rescue recombinant viruses Ad-mda7, AdLuc and AdCMVpA. Plaques were picked, virus stocks were grown and their genomes were confirmed as correct by PCR/restriction analysis and sequencing. Viruses were propagated in 293 cells and purified by HPLC.

f. Transduction and Cell Proliferation studies

Cancer or normal cell lines used in this study are infected with Ad-mda7 (with either AdCMVpA or AdLuc as controls) in increasing MOIs (viral particles/cell; 0, 100, 250, 500, 1000, 2500, 5000, 10000 vp/cell increasing concentrations). Cells were either plated at 500-2000 cells/well in 96-well format for Tritiated thymidine incoporation-Cell Proliferation Assay or plated at $10^5$-$10^6$ cells/well in a 6 well plate for protein expression or Apoptosis assays or plated at $10^4$ cells/well for Alamar-blue assay.

For infection Ad-mda7 or AdLuc (or AdCMVpA) were used at increasing MOIs (based on viral particles/cell; MOI ranged from 0-10,000 viral particles/cell). For tritiated thymidne /apoptosis and protein expression and alomar assays, cells were analyzed 3 and 5 days post-infection

g. Tritiated Thymidine Assay

instruction for Apoptosis using the Chromogenic TUNEL-peroxidase assay ("*In Situ* Death Detection Kit, POD", Boehringer Mannheim).

j. Annexin V Assay

Cancer cells were also analyzed for Apoptosis, post-Ad-mda7 treatment, by ApoAlert Annexin V-FITC kit (CLONTECH). After induction of apoptosis in cells, phosphatidylserine (PS), which is predominantly located on the inner leaflet of the plasma membrane, is rapidly translocated to the outer leaflet via a flippase mechanism. In the presence of $Ca2^+$, annexin V binds PS with high affinity and FITC conjugated to Annexin help to pinpoint apoptotic cells both via flourescent microscopy and FACs analysis.

k. DNA staining with Propidium Iodide (PI)

For determiniNg cells at different stages of cell cycle, Ad-mda7 infected Cancer cells were prepared as a single cell suspension of 1-2 x $10^6$ cells/mL of PBS. After the cells are fixed with cold 70% ethanol for 2 hours, the cells are centrifuged, and the fixative decanted, and washed 2x with PBS and then stained with Propidium Iodide working solution which included PI at 50µg/mL and RNAse at 20µg/mL in PBS. Treated cells were then analyzed by FACS.

l. Tumor Xenograft models

Tumor cells are plated at a density of approximately 20-40% confluency in 150 $mm^2$ dishes in RPMI/10% FBS media supplemented with penicillin, streptomycin and fungizone, and grown in 5% $CO_2$ at 37°C until approximately 80% confluent. Cells are washed twice in PBS, trypsinized, and counted. Cells are diluted to a concentration of $5x10^6$ cells/100ml in PBS. BALB/c nude mice will be injected subcutaneously with $5x10^6$ tumor cells in 100 ml of PBS.

m. Tube formation assay

**TABLE 3: Summary of breast cancer lines used for Ad-mda7 studies.**

| Cell Line | Tumor type | p53 status | Source |
|---|---|---|---|
| ***Breast Cancer*** | | | |
| (1) T47D | ductal carcinoma | L194F | ATCC |
| (2) MCF-7 | carcinoma | wt | ATCC |
| (3) MDA-MB-361 | adenocarcinoma | wt | ATCC |
| (4) MDA-MB-231 | adenocarcinoma | R280K | ATCC |
| (5) MDA-MB-468 | adenocarcinoma | R273H | ATCC |
| (6) SKBr-3 | adenocarcinoma | Mut | ATCC |
| (7) BT-20 | carcinoma | Mut | ATCC |
| ***Normal*** | | | |
| (1) MJ90 | fibroblast | wt | Smith lab |
| (2) HUVECs | endothelium | wt | Clonetics |
| (3) HMECs | mamm. epithelium | wt | Clonetics |

FIG. 7 illustrates the high levels of apoptosis (as measured by Annexin V staining) induced in breast cancer cell lines by Ad-mda-7. Annexin V staining identifies cells in early and mid-stages of apoptosis, whereas the TUNEL assay detects DNA cleavage products, one of the final stages of apoptosis. TUNEL assays performed on MCF-7 cells infected with Ad-mda7 confirmed that these cells are killed via apoptotic pathways. Ad-CMVp(A) or Ad-luc control vectors were ineffective at inducing apoptosis.

The two cell lines that demonstrated the highest sensitivity to Ad-mda7 were T47D (p53-mutant) and MCF-7 (p53 wild-type) (FIG. 2A and 2B). The Ad-mda7 concentration needed to inhibit growth by 50% ($IC_{50}$) of the T47D or MCF-7 cells averaged 500 and 1500 vp/cell, respectively (Table 4). Also included in FIG. 3 (Panels A and B) are representative experiments using MDA-MB-361 and BT-20 cells. These two cell lines also showed marked sensitivity to Ad-mda7 infection. Table 4 summarizes the responsiveness of breast cancer cells to Ad-mda7 infection (as determined by a

3. Ad-mda7 Kills Colorectal Cancer Cells and Induces Apoptosis

Six colorectal cancer lines (DLD-1, SW-620, SW-480, HT-29, HCT-116, LS174T) were infected with Ad-mda7. All of these cell lines were effectively growth inhibited by Ad-mda7 transduction, with SW620, DLD-1 and SW-480 being the most sensitive. SW620 cells treated with Ad-mda7 at variying MOIs is shown in FIG. 5A, while DLD-1 cells are shown in FIG. 5B. Cell proliferation, as determined by $^3$H-thymidine incorporation assay, demonstrated an $IC_{50}$ that averaged 1000 vp/cell in the more sensitive cell lines to 2000 vp/cell in the other less-sensitive cell lines. The DLD-1 cell line was infected with Ad-mda7 at 1000 and 5000 vp/cell, using uninfected cells and Ad-Luc as controls. Forty-eight hours later the transduced cells were analyzed for apoptosis using Annexin V staining in conjunction with FACS analysis. Neither the uninfected or AdLuc-infected (5000 vp/cell) cells showed signs of apoptosis, whereas Ad-mda7 infected cells exhibited approximately 26% apoptotic cells at 1000 vp/cell and 58% apoptotic cells at 5000 vp/cell (FIG. 8).

4. Ad-mda7 Infection in Normal Cells

Three normal human cell lines (MJ90 fibroblasts, HUVEC endothelial cells and human mammary epithelial cells) showed no growth inhibition when infected with Ad-mda7. The primary fibroblast cell line MJ90 showed overlapping growth curves when treated with Ad-mda7 or Ad-luc control vector (FIG. 6A). HUVEC and human mammary epithelium cells showed similar results (FIG. 6B).

5. Protein Analyses

Cell lysates obtained from Ad-mda7 transduced cancer cell lines were size fractionated by SDS-PAGE followed by western-blot analysis using a rabbit anti-MDA7 antibody. The migration of the MDA-7 protein was consistent with an approximate size of 23 kD, however, an additional band at 17 kD was also observed. A Western blot analysis of H1299 (lung cancer) and DLD-1 (colorectal cancer) cell lines was performed after Ad-mda7 and Ad-luc infection. Two bands at approximately 23 and 17 kD were observed. Similar molecular weight size bands were also seen in breast cancer lines

whereas Ad-mda7 infected cells exhibited approximately 26% apoptotic cells at 1000 vp/cell and 58% apoptotic cells at 5000 vp/cell. Ad-mda7 caused rapid induction of apoptosis (FIG. 9). Two cell lines representing NSCLC and colorectal cancer are shown. Substantial levels of apoptosis were evident as soon as 12 hours post-infection with Ad-mda7, and increased over the next few days. The demonstration of apoptosis as soon as 12 hr post-infection is notable as immunoreactive MDA-7 protein is just detectable at 12 hr and, generally, does not peak until 24-48 hr post-infection. Ad-p53 can also cause rapid induction of apoptosis, however, other tumor suppressors, such as p16 or PTEN tend to cause apoptosis only after 2-3 days post infection with the Ad expression vector.

7. Ad-mda7 Increases Bax Protein Levels in Lung, Breast and Colorectal Cancer Lines

Regulation of programmed cell death relies on the interaction between signaling pathways that either promote or inhibit apoptosis (Reed, 1997; White, 1996). The bcl-2 family members (bcl-2, bcl-w, bax, bad, bak, bcl-xs) play an important role in apoptotic signaling (Sedlak *et al.*, 1995; Reed *et al.*, 1996). Using Western blot analysis in conjunction with an anti-bax antibody it was determined that Ad-mda7 infection upregulated the BAX protein in T47D, DLD-1, A549 and H460 cells. Western blot analysis of lysates prepared 24 hours after infection with 30 to 150 pfu/cell of Ad-mda7 demonstrated increased expression of BAX in all cell lines tested. For example, upregulation of BAX in Ad-mda7 infected T47D cancer cell line was observed by Western blot analysis. Cells were infected with Ad-mda7 and analyzed for MDA-7 and BAX protein expression. Ad-mda7 increased BAX expression in T47D, as was observed with the other cell lines.

8. Endogenous Expression of Mda-7 in Cancer and Normal Cells

Of the more than 50 tumor cell lines evaluated for endogenous Mda-7 protein expression, only two, DLD-1 (colorectal) and LnCap (prostate) were weakly positive. Studies are underway to look at mda-7 mRNA in the various cancer lines. Table 5 is a list of some of the cancer lines used in the Ad-mda7 studies and their endogenous MDA-

expression is positively regulated by wild-type p53 (Han *et al.*, 1996), the ability of MDA-7 to induce BAX appears to be independent of p53 since BAX up-regulation is observed in p53-mutant (DLD-1, T47D) and p53-wild-type (H460). It is interesting to note that MDA-7 was able to effectively induce apoptosis in the MCF-7 breast cancer cells that are devoid of caspase 3, one of the several caspases involved in the down-stream apoptotic events.

that Ad-mda7 treatment of H460 cells resulted in high levels of protein production (verified by Western blot analysis) and that the protein appeared to on the cell surface.

2. Confocal Microscopy Studies

To confirm and extend the results shown in FIG. 10, confocal microscopic analyses were performed on various cell lines (H460, H1299, T47D and DLD-1 cells) to determine sub-cellular distribution MDA-7 protein after Ad-mda7 treatment. Background staining in untreated or Ad-luc-treated cells was low and diffuse. The background is believed to be due to the anti-MDA-7 reagent being a polyclonal antiserum. However, highly specific staining was observed when cells were treated with Ad-mda7. At low MOIs, distinct membrane staining was observed with punctate staining in the cytoplasm. At higher MOIs, the punctate staining and membrane staining were reproduced and more intense. The pattern of staining was suggestive of a secreted protein, with the punctate staining representing protein trafficking and release at the plasma membrane. Similar observations were observed in the other cell lines

In additional confocal microscopy experiments, cancer cell lines were treated with Ad-mda7 and analyzed for apoptosis (Annexin V staining), DNA content (Hoechst), Ca2+ influx/eflux (Fluo 3, Molecular Probes) and mitochondrial integrity (MitoTrack, Molecular Probes). The protocols used were those established in the Confocal Microscope Facility, UTHSC, Houston, TX.

Confocal microscopic studies of H460 and MCF-7 cells were done. They show a composite of individual microscopic fields: (1) denotes surface expression of MDA-7 (red surface and punctate staining), (2) showing apoptosing cells (polarized green staining), (3) Hoechst staining to identify nuclei (blue) and (4) composite of (1) (2) and (3).

Calcium and mitochondrial staining was done in Ad-mda7- or Ad-luc control-transduced cells. Cells were plated on laminin-coated cover-slips and treated with

Endoglycosidase treatment suggests that soluble MDA-7 protein is glycosylated. Using various glycosidases, especially Endo F, a lower molecular weight band is also observed (which is approximately the same size as the MDA-7 protein band observed in cell lysate.

3. Inhibition of Glycosylation and Secretion of MDA-7 Protein

Two antibiotics, Tunicamycin and Brefeldin A, have been used to provide a more detailed characterization of the secretion of soluble MDA-7. N-linked glycosylation plays an important role in a protein's ultimate processing, whether it is sorted to a lysosomal pathway, or translocated to the cell surface or secreted. Using Tunicamycin, the N-linked glycosylation process in the golgi apparatus can be inhibited, thus inhibiting protein secretion or other sugar-dependent sorting processes. Brefeldin A is a fungal metabolite (macrocyclic lactone) which exhibits a wide variety of antibiotic activities. Brefeldin A reversibly inhibits the intracellular translocation of proteins (during transport of protein to the cell surface for secretion or expression. Both Tunicamycin and Brefeldin A effectively inhibit the secretion of soluble MDA-7 protein. Therefore, intracellular processing and glycosylation appear to be required for MDA-7 secretion.

4. Secreted MDA-7 Protein Induces Killing in Cancer Cells

The secreted form of MDA-7 (sMDA-7) was produced using various cell lines and evaluated for anti-tumor activity. A representative experiment is shown in FIGS. 11A and 11B. Soluble MDA-7 was analyzed for its anti-proliferative effects on H1299 cells. Briefly, H1299 cells were plated at cell density of $10^3$ cells/chamber in Nunc chamber slides. 24 hours later, the cells were challenged with supernatants obtained from H1299 cells transduced with either Ad-mda-7 or Ad-luc (at 1000 vp/cell infection). Ad-mda7 and Ad-luc viruses were also used as additional controls. The soluble protein supernatants (500 uL total volume, different dilutions) were applied to naïve H1299 cells and 24 hours later an additional 0.5mL of 10% FBS in DMEM was added. After 24 and 48 hours, the cells were microscopically examined for viability using the trypan blue

## EXAMPLE 5: ACTIVATION OF CASPASE CASCADE BY AD-MDA7

1. Material and Methods

a. Cell Culture

Human non-small cell lung carcinoma cells A549, H460, H1299, human prostate cancer cells DU145, and human breast cancer cells MCF-7 were obtained from the American Type Culture Collection (ATCC, Bethesda, MD). All cells were maintained in DMEM medium containing 10% of Fetal Bovine Serum, antibiotics and L-glutamine. Normal human bronchial epithelium cells (NHBE cells) were obtained from Clonetics Inc (Clonetics Inc., Walkersville, MD) and maintained according to the manufacturer's instructions.

The cells were verified to be free of mycoplasma and used in the log phase of growth. Cells were routinely harvested with 0.125% Trypsin –1.3 mM EDTA (GIBCO).

b. Construction of Recombinant Adenoviral Vector

Same as described above.

c. Determination of Cell Growth Rate

Cancer or normal cell lines used in this study were plated in 12-well dishes with 2 x $10^4$ cells in each well. Cells were infected with Ad-mda7, with Ad-Luc controls (5000 viral particles/cell), or with PBS as an additional control. Cells were harvested by trypsinization, diluted with trypan blue (GIBCO) and the numbers of viable cells were counted on a hemocytometer. In addition, inhibition of cell growth was assayed by XTT assay as per the manufacturer's guidelines (Cell Proliferation Detection Kit II, Roche) or by $H^3$-thymidine assay.

d. Cell Cycle Analysis

Fluorescence-activated cell sorter analysis was performed as follows: cells ($5x10^5$/plate) were seeded on 10cm plates and infected with PBS, Ad-mda7 or Ad-Luc at

were homogenized with sonicator for 30 sec and after an hour incubation on ice, cell extracts were spun for 5min at 14000 rpm at 4°C. Cell extracts were collected and stored in –70°C. Protein concentrations of all extracts were determined using the Bio-Rad protein determination kit (Bio-Rad). Each of 50μg protein samples were diluted into 20μl with lysis buffer and 5% of 2-Mercaptoethanol (Bio-Rad) and heated in a water bath at 95°C for 5min. Then protein extracts were separated on a 10% SDS-PAGE gel in a vertical slab gel electrophoresis cell (Bio-Rad). Proteins were transferred from gel to nitrocellulose membrane (Hybond-ECL membranes, Amarsham International, Little Chalfont, England). Proteins were blocked in a blocking solution (5% dry milk and 0.3% Tween 20 in PBS) for one hour at room temperature. Membranes were incubated with primary antibody and then horse raddish peroxidase labeled secondary antibodies followed by application of Enhanced Chemiluminescence Western Blotting Detection System (Amersham) for 30 seconds. Proteins were visualized on Amersham Hyperfilm enhanced chemiluminescence film using exposure time varying 30 seconds to 30minutes.

2. Inhibition of Cell Proliferation by Overexpression of MDA-7

To detect MDA-7 expression in cells, A549, H1299, H460, and NHBE cells were infected with 5000vp/cell of Ad-mda7. Forty-eight hours later cells were fixed and stained with anti-MDA-7 antibody. Uninfected cells were stained with the same antibody as controls. High level of MDA-7 expression was observed in cytoplasm of cells, while no stained cells were seen in uninfected controls (FIG. 14).

A549, H1299, H460, and NHBE cells were prepared in 12 well plates and treated with Ad-mda7, Ad-Luc, or PBS. The numbers of viable cells were counted from day 1 to day 5 after treatment. Infection with Ad-mda7 significantly suppressed cell proliferation in all the tumor cell lines as compare to PBS or Ad-Luc controls.

DU-145 cells were sensitive to Ad-mda7 infection and displayed growth arrest and apoptosis. p53 and bax are up-regulated by Ad-mda7 in p53 wild-type tumor cells. In addition, caspases 3 and 9 and PARP are activated by Ad-mda7. Normal cells do not exhibit alterations in apoptotic mediators.

4. Activation of Caspase Cascade and Cleavage of PARP

Western blots demonstrated activation of the caspase cascade by Ad-mda7 infection. The proforms of caspase-9 and caspase-3 were cleaved and converted to the activated/ cleaved forms 48 hrs after Ad-mda7 infection in A549 and H460 cells and after 72hrs in H1299 cells. Cleavage of caspase-8 was demonstrated after 48 hrs of Ad-mda7 infection in A549 and H460 cells. Poly (ADP-ribose) polymerase (PARP) was cleaved in A549 and H460 cells after 48 hrs in H1299 cells. In Bax-deficient DU145 cells, caspase-9 and caspase-3 were cleaved after 72 hrs of Ad-mda7 infection.

## EXAMPLE 6: *IN VIVO* EFFECTS OF AD-MDA7

1. Materials and Methods

a. Cell culture

Human non-small cell lung carcinoma cells A549 and H1299 were obtained from the American Type Culture Collection (ATCC, Bethesda, MD). All cells were maintained in RPMI1640 medium containing 10% of Fetal Bovine Serum, antibiotics and L-glutamine. Prior to start of the experiments, the cells were verified to be free of mycoplasma and used in the log phase of growth. Cells were routinely harvested with 0.125% Trypsin –1.3 mM EDTA (GIBCO).

b. Construction of recombinant adenoviral vector

Replication-deficient human type 5 Adenoviral vectors (Ad5) carrying the mda-7 or Luc genes linked to an internal CMV-IE promoter and followed by SV40 polyadenylation (pA) signal have been constructed and will be referred to as Ad-mda7

e. Evaluation of Tumor Growth and Treatments *in vivo*

Prior to the start of all experiments involving subcutaneous tumor growth and treatments, *nu/nu* mice were irradiated (3.5 Gy) using a cesium source to enhance tumor uptake. In all the experiments, $5x10^6$ tumor cells (H1299, A549) suspended in 100 µl sterile phosphate buffered saline (PBS) were injected into the right dorsal flank. When the tumor had reached a size of 50-100$mm^3$, animals were randomized into three groups (n = 8 animals/group) and treatment initiated as follows. Group 1 received no treatment, Group 2 received Ad-Luc ($5 \times 10^9$ vp / dose) and Group 3 received Ad-mda-7 ($5 \times 10^9$ vp / dose) every alternate day for a total of three doses. Intratumoral injections were performed under anesthesia using methoxyflurane (Schering Plough, Kenilworth, NJ) as per institutional guidelines. Tumor measurements were recorded every other day without knowledge of the treatment groups, and the volume was calculated using the formula V (mm3) = a x $b^2$ / 2, where "a" is the largest dimension and "b" is the perpendicular diameter. Antitumor efficacy data are presented as cumulative tumor volumes for all animals in each group to account for both size and number of tumors.

f. Immunohistochemical Analysis

Tumors established subcutaneously in nude mice were obtained and fixed in 10% buffered formalin, paraffin embedded and cut as 4 µm thick sections. Sections were stained for mda-7 gene expression. Briefly, tissue sections were treated with 0.3% $H_2O_2$ in methanol for 30 minutes to block endogenous peroxidase activity and were subsequently incubated with normal goat serum for 30 minutes at room temperature. Following incubation, slides were treated with rabbit polyclonal anti-MDA-7 antibody (1:5000 dilution) for 60 minutes. After 30 minutes incubation with anti-rabbit secondary antibody (provided with ABC kit, Vector) protein expression of MDA-7 in tissues were detected with DAB by enhancement with avidin-biotin reaction ABC kit. The slides will be counterstained with hematoxylin and then mounted with Aqua-mount (Lerner Labs., Pittsburgh, PA). The number of tumor cells staining positive for MDA-7 were analyzed

MDA-7 gene expression *in situ* results in apoptotic cell death through caspase-3 and Apo2/TRAIL activation. To understand the mechanism of tumor inhibition mediated by mda-7, subcutaneous tumors harvested at 48 hours following the last treatment were analyzed for apoptotic tumor cell death by TUNEL staining. Tumors from control mice that were either untreated or treated with Ad-Luc showed minimal apoptotic cell death while tumors from animals treated with Ad-mda-7 demonstrated extensive apoptosis.

Since apoptosis is mediated by activation of caspases, tumor tissues were examined for caspase-3, a downstream caspase. Activated form of caspase-3 was observed in tissues treated with Ad-mda-7 while no caspase-3 activation was observed in the tissues from control mice. Similarly, activation of Apo2/TRAIL was observed in tumors expressing mda-7. In contrast, TRAIL expression was not observed in tumors that were not treated or treated with Ad-luc.

3. MDA-7 Expression Results in Upregulation of Costimulatory Molecules

The ability of dying tumor cells *in situ* to activate costimulatory molecules, B7 and ICAM, was investigated. Subcutaneous tumors injected with Ad-MDA7 or Ad-Luc were harvested 48 hrs following the last dose and analyzed by immunohistochemistry. Expression of B7 (7.1 and 7.2) and ICAM was observed in tumors expressing MDA-7 while no expression was observed in tumors treated with Ad-Luc.

4. Expression of MDA-7 in *in situ* Tumor Inhibits Angiogenesis

To further determine the tumor suppressive effects of mda-7, subcutaneous tumors were analyzed for CD31 expression, a marker frequently used to identify angiogenesis in tumors. Subcutaneous tumors treated with Ad-mda-7 demonstrated fewer numbers of blood vessels when compared to tumors treated with Ad-luc or no treatment groups.

tumor sections with high numbers of capiallaries and small venules will be found by scanning the sections at low power (x40 and x100). In these areas individual vessels will be counted in x200 magnification fields, and average scores recorded for the treated and untreated tumor samples. This method has been used to compare blood distribution and density in human xenografts in nude mice (Yoneda *et al.*, 1998).

## EXAMPLE 8: Modulation Of Growth Factors During Ectopic Expression Of Mda-7

Because it has been hypothesized that MDA-7 has an autocrine/paracrine activity, the effect of Ad-mda7 on melanoma cells will be evaluated with respect to the secretion of factors involved in the progression of melanoma. ELISA assays will be used to address the release of these soluble mediators, such as different types of TGF-β1, IL-8, IL-10, and bFGF. Melanoma cells lines and normal cells will be treated with Ad-mda7, Ad-luc, or diluent control and then monitored for modulation of growth factor levels in culture supernatant after 24-48 hours. Immunoblotting on the lysates may also be performed at various times post-treatment.

## EXAMPLE 9: AD-MDA7 ENHANCES ACTIVITY OF HERCEPTIN

The breast cancer SkBr3 (Her2+) and MCF-7 (Her2-) cell lines were both obtained from ATCC. Cells were plated at a density of 1000 cells/well in Nunc 2-chamber slides and propagated in DMEM medium with 10% FBS. The following day, the cells were left untreated or treated with Ad-mda7 at (increasing MOIs: 0, 500, 1000 and 2000 vp/cell) without (M series) or with Herceptin (M+H series) at a final concentration of 1 μg/mL. The cells were washed after 3 hours and growth media (with or without Herceptin, as indicated) was replaced. Three days later viable cells were counted using the trypan blue exclusion assay (average of 3-4 fields) and plotted as shown in FIG. 15. Herceptin alone yields approximately 12% dead cells in both cell lines. However, Ad-mda7 appeared to enhance the killing effect of Herceptin in breast cancer cell lines.

**Tube Formation Assay**. Human umbilical vein endothelial cells (HUVECs; Clonetics) were seeded on 1% gelatin-coated plates and incubated at 37°C for 24 hours. After incubation, cells were infected for 1 hour with Ad-*luc* or Ad-*mda7* at 10,000 vp/cell in serum-free medium. Cells exposed to medium alone served as negative controls while cells exposed to Suramin (50 μM) served as positive controls. After a 48-hour incubation period (37°C in serum-containing medium), infected cells were harvested, counted, and added to Matrigel-coated 24-well plates in triplicate ($1.2 \times 10^5$ cells per well). Twenty-four hours later, cells were fixed with 10% buffered formalin and examined for differentiation (tube formation) by using an Olympus IX-70 inverted bright-field microscope at 4X and 10X magnification.

**Evaluation of Tumor Growth *in vivo***. Before the start of all experiments involving subcutaneous tumor growth and treatments, *nu/nu* mice were irradiated (3.5 Gy) from a cesium source to enhance tumor uptake. In all the experiments, $5 \times 10^6$ tumor cells (A549, H1299) suspended in 100 μl sterile PBS were injected subcutaneously into the right dorsal flank. When the tumor had reached a size of 50-100 $mm^3$, animals were randomized into three groups (n = 8 animals/group) and treatment was initiated as follows. Group 1 received no treatment; Group 2 received Ad-*luc* ($5 \times 10^9$ vp/dose); and Group 3 received Ad-*mda7* ($5 \times 10^9$ vp/dose); all treatments were given every other day for a total of three doses. Intratumoral injections were performed under methoxyflurane anesthesia (Schering Plough, Kenilworth, NJ) per institutional guidelines. Tumor measurements were recorded every other day without knowledge of the treatment groups, and tumor volumes were calculated by using the formula $V\ (mm_3) = a \times b^2/2$, where "a" is the largest dimension and "b" is the perpendicular diameter (Georges *et al.*, 1993). Antitumor efficacy data are presented as average tumor volumes for all animals in each group to account for both size and number of tumors.

**Immunohistochemical Analysis**. Xenograft tumors established in nude mice were harvested and fixed in 10% buffered formalin, embedded in paraffin, and cut in 4-μm sections. Briefly, tissue sections were treated with 0.3% $H_2O_2$ in methanol for 30

observed in NHBE cells infected with Ad-*mda7* or Ad-*luc*. Furthermore, analysis of the effects of MDA-7 overexpression on cell proliferation demonstrated significant inhibition of tumor cells with 27% inhibition of H1299 cells and 40% inhibition of A549 cells at seventy-two hours after infection (FIG. 16B). In contrast, NHBE cells showed no growth inhibition.

**MDA-7 overexpression inhibited endothelial cell differentiation *in vitro*.** The ability of Ad-*mda7* to inhibit endothelial cell differentiation was evaluated in HUVEC cells. Ad-*mda7* inhibits endothelial cell differentiation into capillary-like structures (tube-formation). Human umbilical vein endothelial cells (HUVEC) were treated with Suramin, Ad-*luc* (10,000 vp/cell) or Ad-*mda7* (10,000 vp/cell), or not treated. Forty-eight hours later, cells were harvested, mixed with Matrigel, and observed for tube formation. Overexpression of MDA-7 resulted in inhibition of endothelial tube formation, an effect similar to that of Suramin, a known inhibitor of tube formation. In contrast, cells infected with control vector (Ad-*luc*) demonstrated no inhibition of tube formation. That the observed inhibition of tube formation by endothelial cells was due to MDA-7 overexpression and not due to cytotoxicity was determined by trypan blue exclusion assay for cell viability. More than 80% of cells expressing MDA-7 were viable. The inhibition of tube formation suggests that Ad-mda7 may possess antiangiogenic activity in vivo (see below).

***In vivo* evaluation of local tumor growth suppression by *mda-7*.** We assessed the therapeutic effects of intratumoral injection of Ad*mda–7* on A549 and H1299 subcutaneous tumors in nude mice. Mice bearing experimentally induced xenograft tumors (A549 or H1299) were divided into three groups, one receiving no treatment, one treatment with Ad-*luc*, and one treatment with the Ad-*mda7* daily for a total of three doses ($5x10^9$ vp / dose). Significant inhibition of the growth of both H1299 tumors ($p = 0.01$) and A549 tumors ($p = 0.001$) was observed in mice treated with Ad-*mda7* but not in the control groups for either tumor type (FIG. 17).

evaluated. Pre-treatment or non-injected tumors were negative for MDA-7 immunohistochemistry. After Ad-mda-7 injection, high levels of transgenic MDA-7 protein were detected and high levels of apoptosis were observed (FIG. 20). In some patients, MDA-7 protein and TUNEL positivity was detected at the periphery of the tumor (>1 cm from injection). Furthermore, PCR amplification for mda-7 sequences showed high levels of Ad-mda7 DNA in the center of injected lesions (FIG. 21).

## EXAMPLE 12: MDA-7 PROTEIN MAY BE ADMINISTERED AS TREATMENT

HUVEC cells were administered increasing amounts of MDA-7 protein purified from 293-mda7 cells. Doses that were evaluated ranged from 0.5 - 100 ng/ml. The $ED_{50}$ of MDA-7 ranged from 5-50 ng/ml. Endothelial differentiation in the cells was inhibited by MDA-7 protein, but not control cells, based on tube formation.

HUVEC cells were given varying doses of MDA-7 protein purified from 293-mda7 cells (lots 1-6) or from baculovirus exoressing mda-7. A positive control of Ad-mda7 and a negative control of Ad-luc were included in most assays. Ad-mda7 inhibited tube formation and MDA-7 protein also inhibited tube formation at doses as low as 0.5-10 ng/ml (FIG. 22).

## EXAMPLE 13: MDA-7 HAS CYTOKINE ACTIVITY

### 1. Materials and Methods

**Activation of PBMC:** PBMC were isolated from the peripheral blood of normal healthy donors by centrifugation over Histopaque (Sigma, St. Louis, MO). Cells were cultured at a concentration of $1x10^6$ cells/ml in RPMI-1640 based media supplemented with L-glutamine, Hepes, penicillin, streptomycin, and 10% human AB serum (Pelfreez, Brown Deer, WI) for 72 hr in the presence of PHA-P at 5 μg/ml or LPS 10 μg/ml (both from Sigma, St. Louis, MO). Four hours prior to harvest Brefeldin A (BFA, Sigma-Aldrich) was added at a final concentration of 10 μg/ml. The supernatants as well as cells were then harvested.

**Western blotting.** The activated PBMC were washed once in PBS, resuspended in modified RIPA buffer (TBS, pH 7.6, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM sodium fluoride, 0.2 mM aprotinin, 1 mM leupeptin) and rocked at 4°C for 20 minutes. Lysates were cleared by a 30 min centrifugation at 16,000 x g at 4°C. Protein concentrations were determined with the DC Protein Assay (Bio-Rad, Hercules, CA) and samples were boiled for 5 minutes in an equal volume of sample buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol). Samples were separated by SDS-PAGE on a 12% gel and transferred to nitrocellulose. The membrane was blocked for 30 minutes with blocking buffer and incubated in a rabbit polyclonal MDA-7 Ab (Introgen Therapeutics, Houston, TX) in blocking buffer. Subsequently the membranes were washed twice in PBST, incubated at 1:2000 with HRP conjugated goat anti-rabbit secondary Ab. Blots were developed with ECL reagent (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were incubated in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 2% SDS and 100 mM β-mercaptoethanol) for 30 min at 60°C, washed three times with PBST for 10 minutes each, and reprobed with anti-actin antibody (1:1000).

**Purification of human MDA-7.** The full length cDNA of mda-7 was cloned into the pCEP4 FLAG vector (Invitrogen), which uses the CMV promoter to drive mda-7 gene expression. The plasmid was transfected into HEK 293 cells and antibiotic resistant stable subclones were isolated using hygromycin (0.4 ug/mL). Purification of MDA-7 protein was performed using the HEK 293 cell supernatants collected from viable cells in log phase growth. The crude supernatant was determined by ELISA to contain approximately 30 ng/ml MDA-7. No actin was found in the supernatant (data not shown), strongly supporting the premise that the MDA-7 material derived was not from dead cells, but secreted from completely viable and healthy cells. Supernatant containing the secreted MDA-7 was supplemented with protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.5 mM PMSF) and 0.05% sodium azide and were concentrated 10-fold with an Amicon stirred cell on a YM10 membrane. Ten ml aliquots of concentrated

strongly positive, subclass analysis was performed using subsets selected from PHA stimulated PBMC. Positively selected monocytes (3/3 experiments) and CD3+ T cells (6/6 experiments) were routinely negative, but CD56+ and CD19+ subpopulations resulting from the same starting PBMC and separation procedures were unequivocally positive. Membrane staining was most evident in the CD56+ cells, and a granular location in both types of cells was observed.

**MDA-7 can be a secreted protein.** One of the characteristics of a cytokine is its ability to be secreted. Usually, a short stretch of hydrophobic amino acids at the amino terminus of a protein signals and targets it to a secretory pathway. As depicted in FIG. 22A, the mda-7 cDNA sequence contains a leader sequence consisting of 49 amino acids; this is depicted in more detail in the hydrophobicity plot (FIG. 23B). The predicted cleavage site was determined by the von Heijne SignalP predictions program (Nielsen *et al.*, 1997), however, to the best of our available information, this cleavage site in MDA-7 has not been confirmed experimentally. In order to demonstrate secretion of MDA-7 from mammalian cells, stable transfectants of 293 cells containing the human MDA-7 full-length cDNA were generated (Mhashilkar *et al.*, 2001). Supernatants were analyzed for MDA-7 expression by the Western blot and four bands of MDA-7 protein were detected in the culture supernatants of MDA-7 transfected but not untransfected 293 cells. At this time, the molecular nature of the multiple size bands of MDA-7 after secretion is not clear. Based on the amino acid sequence, MDA-7 is expected to have a molecular weight of 18,419 and when containing the leader sequence it is 23,824 kDa (ProtParam tool). As with IL-10, homodimerization of MDA-7 is likely to occur; also the possibility of variable glycosylation for MDA-7 is also a consideration, and both of these postranslational modifications are being investigated. Many cytokines have been demonstrated to be glycosylated to varying degrees (May *et al.*, 1991; Gross *et al.*, 1989).

**MDA-7 protein induces secondary cytokines, inhibited by IL-10.** Another hallmark of the cytokine family is that of belonging in a cascade of additional molecules

production of proinflammatory cytokines. Therefore, we hypothesized that IL-10 and MDA-7 may be antagonists. To test this hypothesis, human recombinant IL-10 was added to the PBMC cultures stimulated by MDA-7. It was found that under the conditions used, IL-10 completely abrogated TNFα and IFNγ induction by MDA-7 and partially blocked IL-6 induction by MDA-7 (FIG. 24). As a positive control, the IL-10 also prevented the production of two of these three cytokines in response to LPS. The lack of IL-10 inhibition of LPS induced IL-6 secretion is probably due to the IL-6 values greatly exceeding the standard curve of the assay. IL-10 also partially inhibited IL-1β and GM-CSF production and completely inhibited IL-12 production (FIG. 25). As with any study using freshly isolated human PBMC there was some variability from donor to donor, but the result of MDA-7 inducing secondary cytokines and inhibition by IL-10 was consistent in all donors tested and all experiments.

**MDA-7 does not appear to function as a growth factor for human PBMC.** Some cytokines can also function as growth factors. Therefore the proliferative simulation function of MDA-7 was addressed using PBMC. IL-10 was included as a negative cytokine control. PHA was used as positive control and induced a robust uptake of thymidine in all three donors. As expected, IL-10 did not induce increased thymidine uptake of PBMC over the course of four day. Our results show that MDA-7 did not induce significant proliferation during 4 days of coculture of the PBMC population in any of the three donors tested. Earlier studies employing recombinant MDA-7 (up to 5 μg/ml) expressed in E. coli or S. cerevisiae also did not show a proliferative response in human PBMC from three donors.

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All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred

## References

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.


U.S. Patent 4,797,368

U.S. Patent 4,870,287

U.S. Patent 5,139,941

U.S. Patent 5,399,363

U.S. Patent 5,466,468

U.S. Patent 5,543,158

U.S. Patent 5,633,016

U.S. Patent 5,641,515

U.S. Patent 5,656,016

U.S. Patent 5,697,899

U.S. Patent 5,739,169

U.S. Patent 5,760,395

U.S. Patent 5,779,708

U.S. Patent 5,770,219

U.S. Patent 5,783,208

U.S. Patent 5,797,898

U.S. Patent 5,798,339

U.S. Patent. 5,801,005

U.S. Patent 5,811,128

U.S. Patent 5,824,311

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**WHAT IS CLAIMED IS:**

1. A method of inhibiting angiogenesis in a patient in need of such treatment comprising administering to the patient a human MDA-7 polypeptide or a nucleic acid expressing the human MDA-7 polypeptide in eukaryotic cells, whereby the MDA-7 polypeptide inhibits angiogenesis in the patient.

2. The method of claim 1, wherein said patient exhibits an angiogenesis-related disease.

3. The method of claim 2, wherein the angiogenesis-related disease is further defined as angiogenesis-dependent cancer, a benign tumor, rheumatoid arthritis, psoriasis, an ocular angiogenic disease, Osler-Webber Syndrome, myocardial angiogenesis, plaque neovascularization, a telangiectasia, hemophiliac joint, angiofibroma, wound granulation, cat scratch disease, an ulcer, an intestinal adhesion, atherosclerosis, scleroderma, or a hypertrophic scar.

4. The method of claim 3, wherein angiogenesis-dependent cancer is further defined as a solid tumor, leukemia, or a tumor metastasis.

5. The method of claim 3, wherein the benign tumor is further defined as a hemangioma, a neuroma, a neurofibroma, a trachoma, uterine fibroid, hamartoma, teratoma, or a pyogenic granuloma.

6. The method of claim 2 wherein the ocular angiogenic disease is further defined as diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, or Rubeosis.

7. The method of claim 1, wherein the nucleic acid is an expression vector.

18. The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered to the patient by continuous infusion.

19. The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered to the patient by intravenous injection.

20. The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered prior to or after surgery.

21. The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered before chemotherapy, immunotherapy, or radiotherapy.

22. The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered during chemotherapy, immunotherapy, or radiotherapy.

23. The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered after chemotherapy, immunotherapy, or radiotherapy.

24. The method of claim 1, wherein the patient is a human.

25. The method of claim 1, wherein the MDA polypeptide comprises amino acids from 1 to 206 of SEQ ID NO:2.

26. The method of claim 1, wherein the MDA polypeptide comprises amino acids from 49 to 206 of SEQ ID NO:2.

27. The method of claim 1, wherein the MDA polypeptide comprises amino acids from 75 to 206 of SEQ ID NO:1.

38. The method of claim 36 wherein a chemotherapeutic agent is administered after administration of the MDA-7 polypeptide or the nucleic acid molecule.

39. The method of claim 36, wherein the chemotherapeutic agent is a DNA damaging agent.

40. The method of claim 39, wherein the DNA damaging agent is gamma-irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, adriamycin, 5-fluorouracil (5FU), etoposide (VP-16), camptothecin, actinomycin-D, mitomycin C, cisplatin (CDDP), or hydrogen peroxide.

41. The method of claim 38, wherein the chemotherapeutic agent is a cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, bisulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen, taxol, transplatinum, 5-fluorouracil, vincristin, vinblastin, methotrexate, or analog or derivative variant thereof.

42. The method of claim 36, wherein the nucleic acid is comprised within a viral vector.

43. The method of claim 36, wherein the nucleic acid is comprised in a lipid composition.

44. A method for promoting an immune response in a patient comprising providing to the subject an amount of an MDA-7 polypeptide effective to induce an immune response in the patient.

sequence encoding the antigen, wherein the nucleic acid sequence is under the transcriptional control of a promoter.

56. The method of claim 44, wherein the MDA-7, antigen, or both are provided to the patient more than one time.

57. The method of claim 44, wherein the MDA-7, antigen, or both are provided to the patient intravenously, directly, intraperitoneally, regionally, systemically, or orally.

58. The method of claim 44, wherein the MDA-7 and antigen are provided to the subject at the same time.

59. A method of inducing expression of IL-6, IFNγ, or TNFα in a cell comprising administering to the cell an effective amount of an MDA-7 polypeptide or a nucleic acid expressing the MDA-7 polypeptide.

60. The method of claim 59, wherein expression of IL-6 is induced.

61. The method of claim 59, wherein expression of TNFα is induced.

62. The method of claim 59, wherein expression of IFNγ is induced.

63. The method of claim 59, wherein the cell is in a patient.

64. A method of reducing cell damage from chemotherapy or radiotherapy in a cancer patient comprising administering to the patient an effective amount of a human MDA-7 polypeptide or a nucleic acid expressing the human MDA-7 polypeptide.

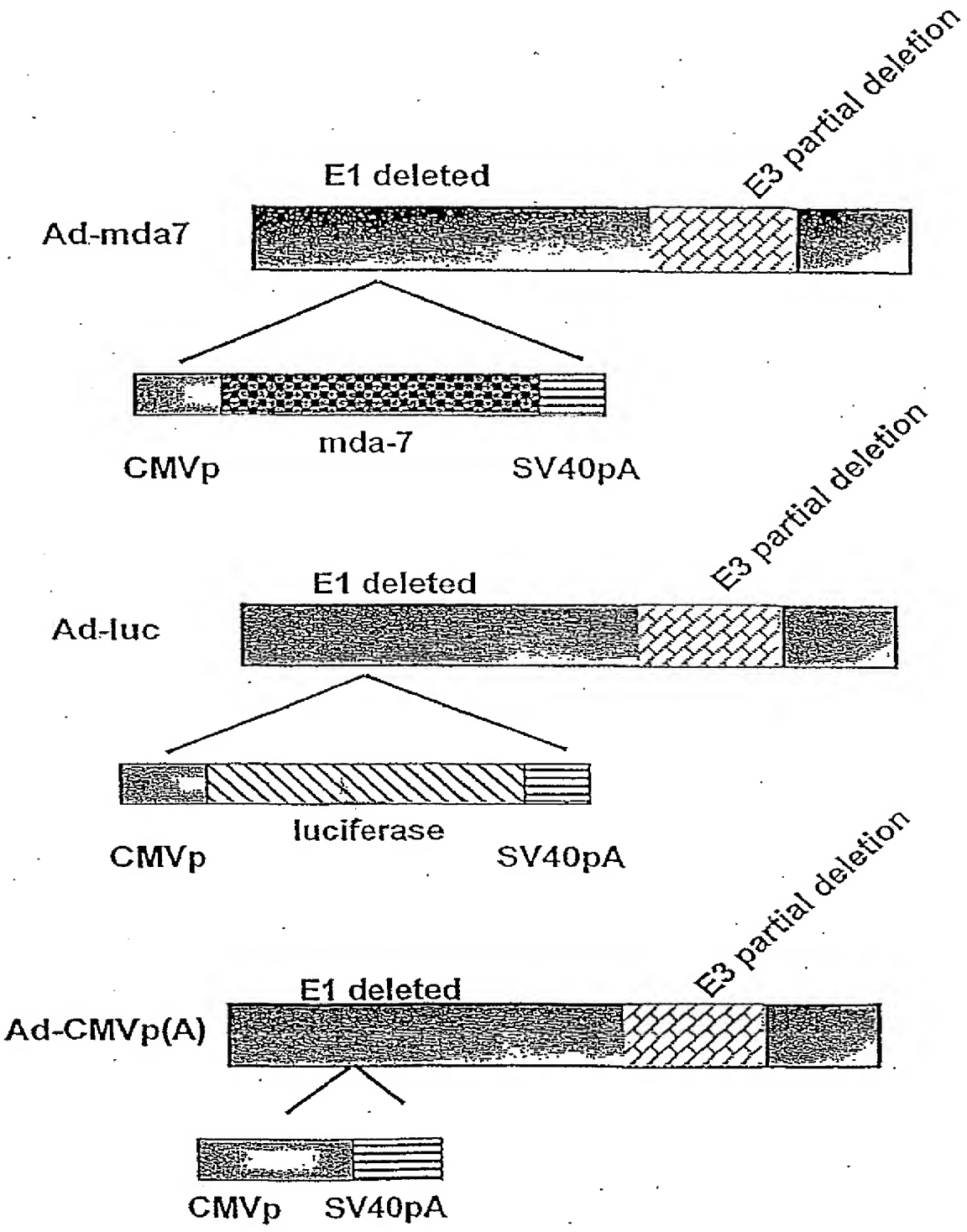
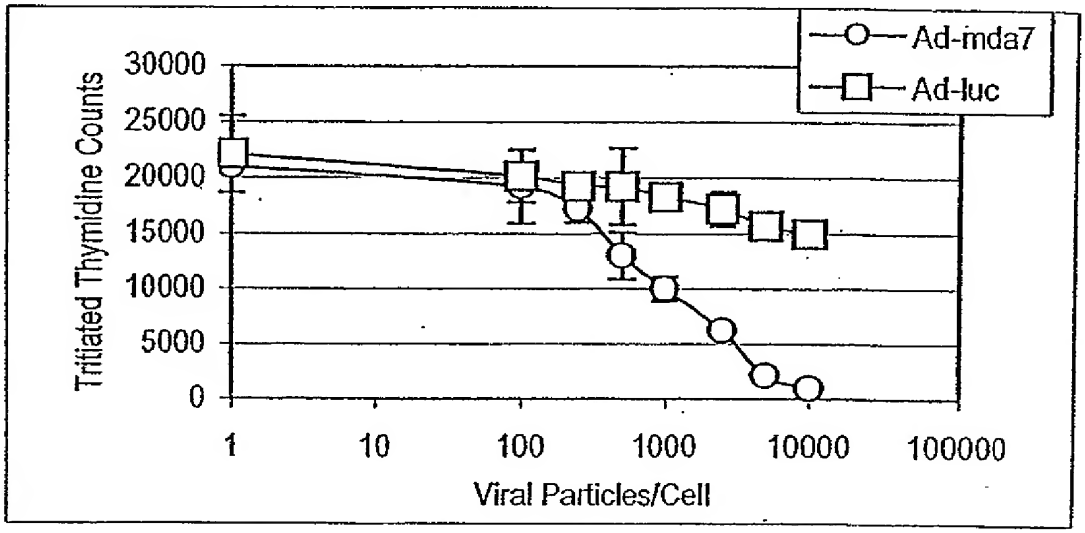

E1 deleted
E3 partial deletion
Ad-mda7
mda-7
CMVp
SV40pA
E1 deleted
E3 partial deletion
Ad-luc
luciferase
CMVp
SV40pA
E1 deleted
E3 partial deletion
Ad-CMVp(A)
CMVp
SV40pA

FIG. 1

A MDA-MB-361 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell)




B BT-20 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell)

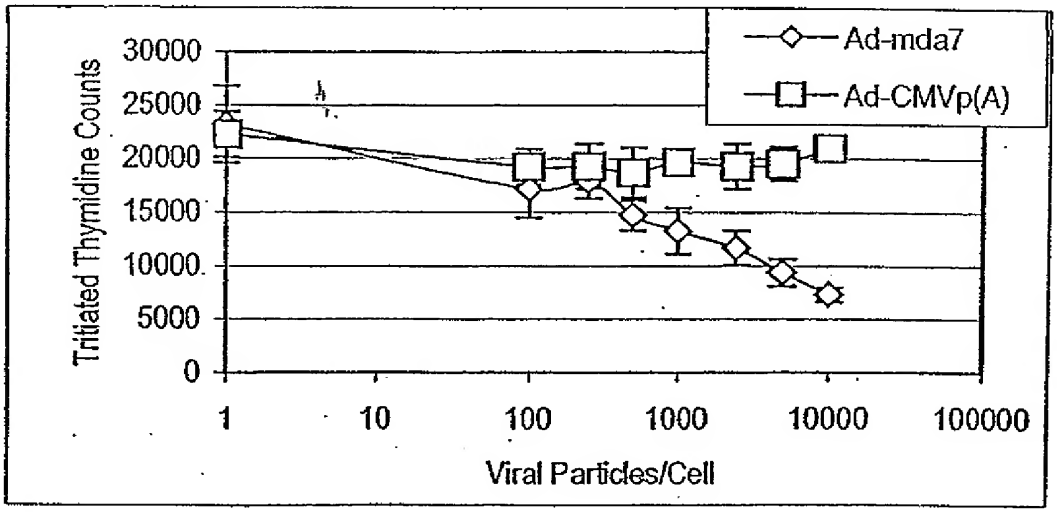
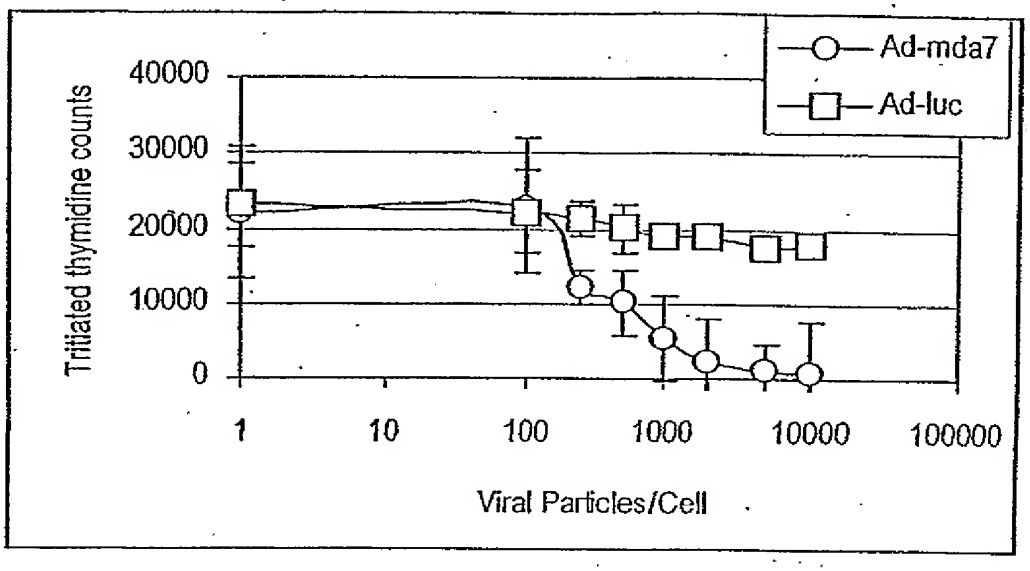



FIG. 3

A SW620 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell)




B DLD-1 cells treated with Ad-mda7 at varying MOIs (Viral particle/cell)

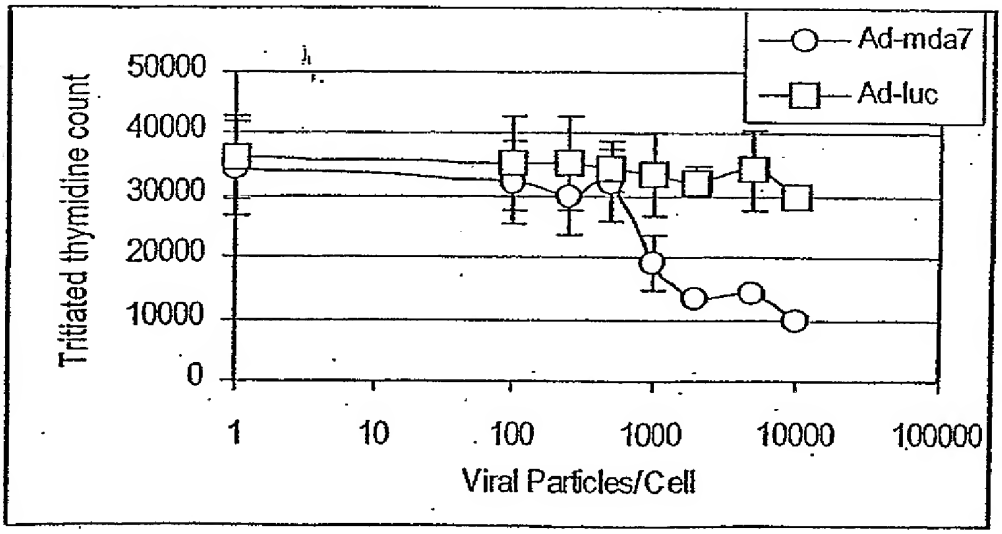



FIG. 5

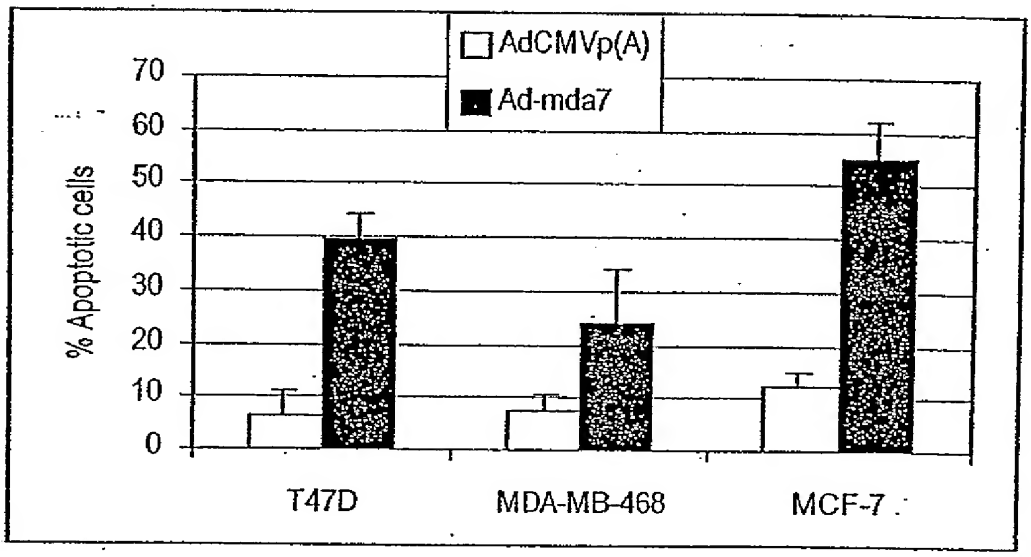

AdCMVp(A)
Ad-mda7
% Apoptotic cells
70
60
50
40
30
20
10
0
T47D
MDA-MB-468
MCF-7

FIG. 7

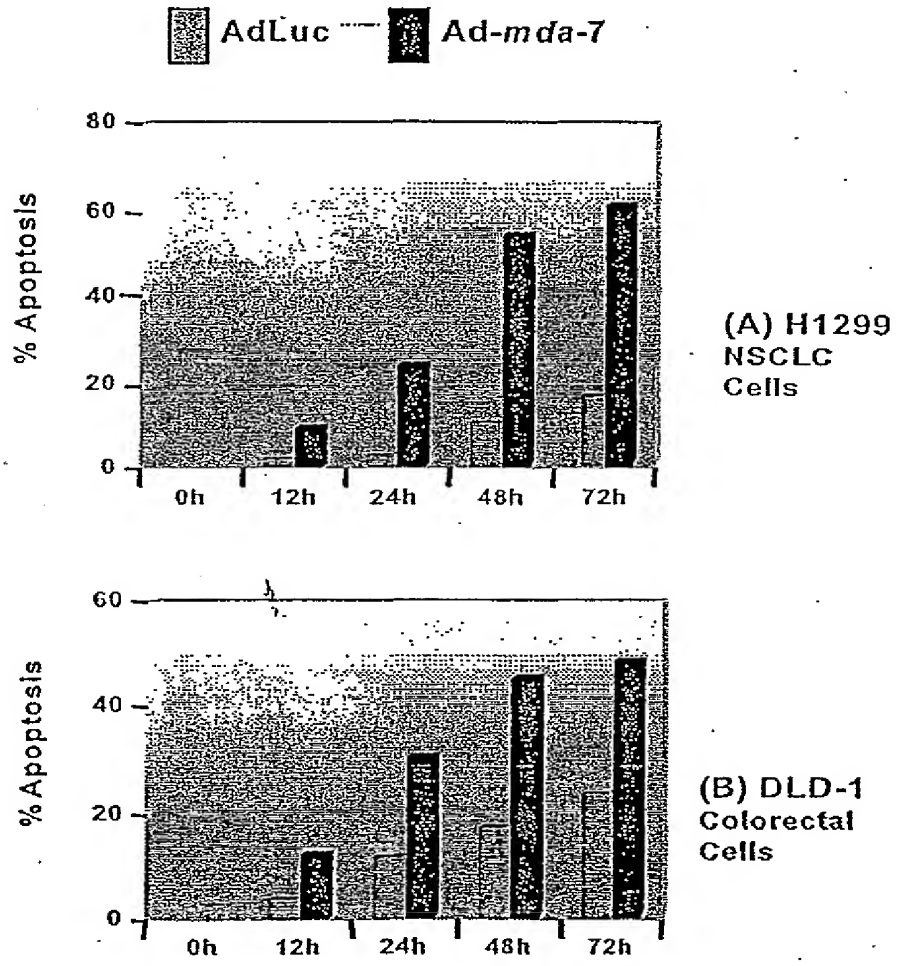
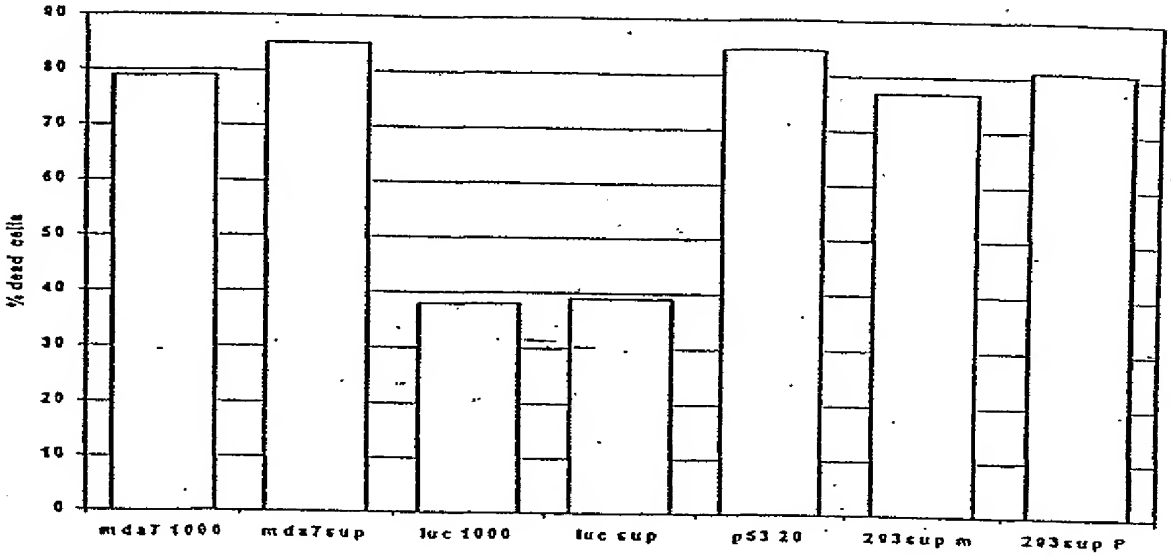

AdLuc
Ad-mda-7
% Apoptosis
80
60
40
20
0
0h
12h
24h
48h
72h
(A) H1299 NSCLC Cells
% Apoptosis
60
40
20
0
0h
12h
24h
48h
72h
(B) DLD-1 Colorectal Cells

FIG. 9

A


% dead cells
0
10
20
30
40
50
60
70
80
90
mda7 1000
mda7sup
luc 1000
luc sup
p53 20
293sup m
293sup P

B

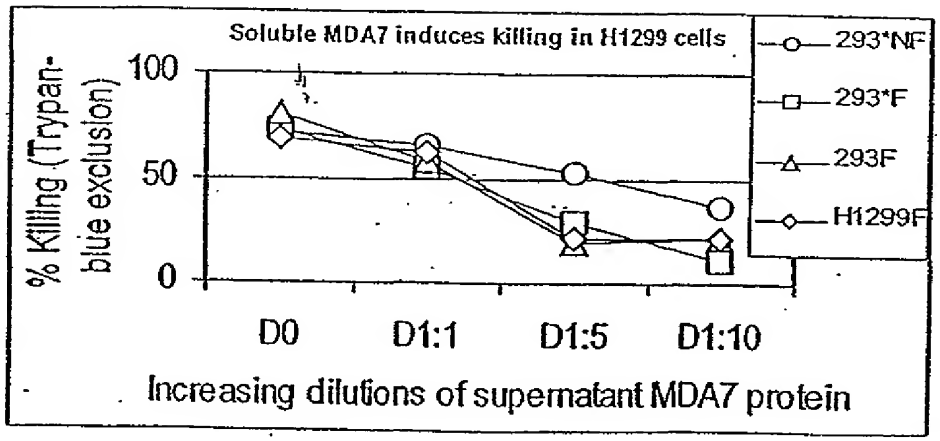
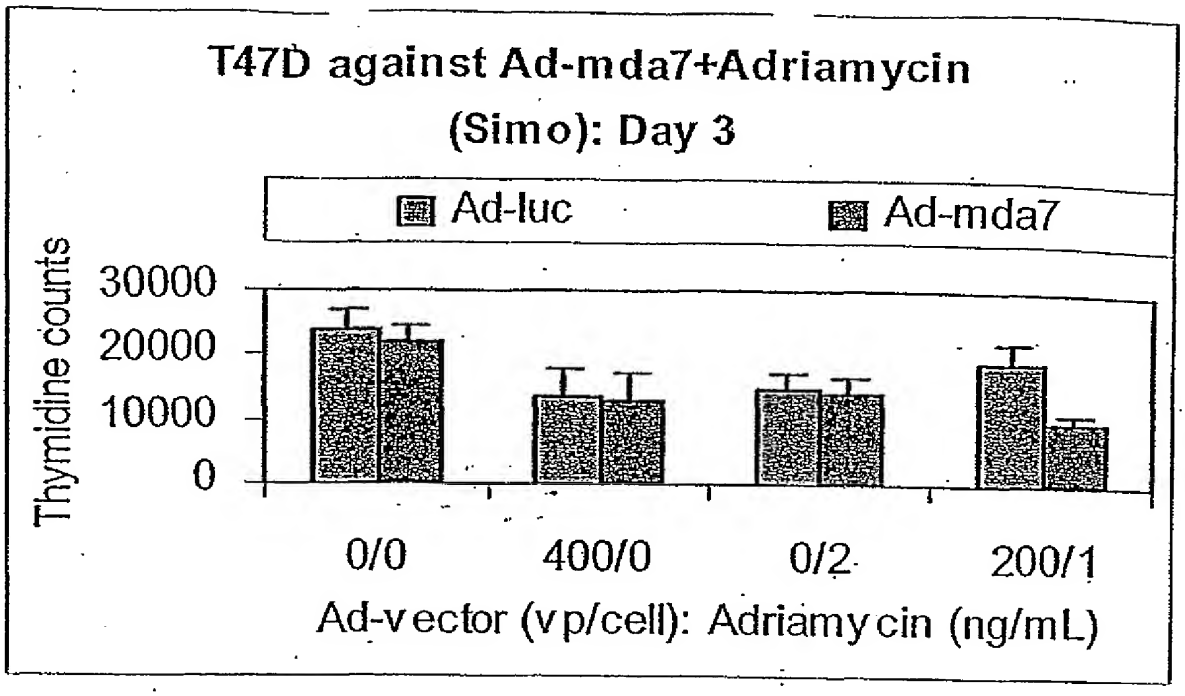

Soluble MDA7 induces killing in H1299 cells
% Killing (Trypan-blue exclusion)
100
50
0
D0
D1:1
D1:5
D1:10
Increasing dilutions of supernatant MDA7 protein
293*NF
293*F
293F
H1299F

FIG. 11




B

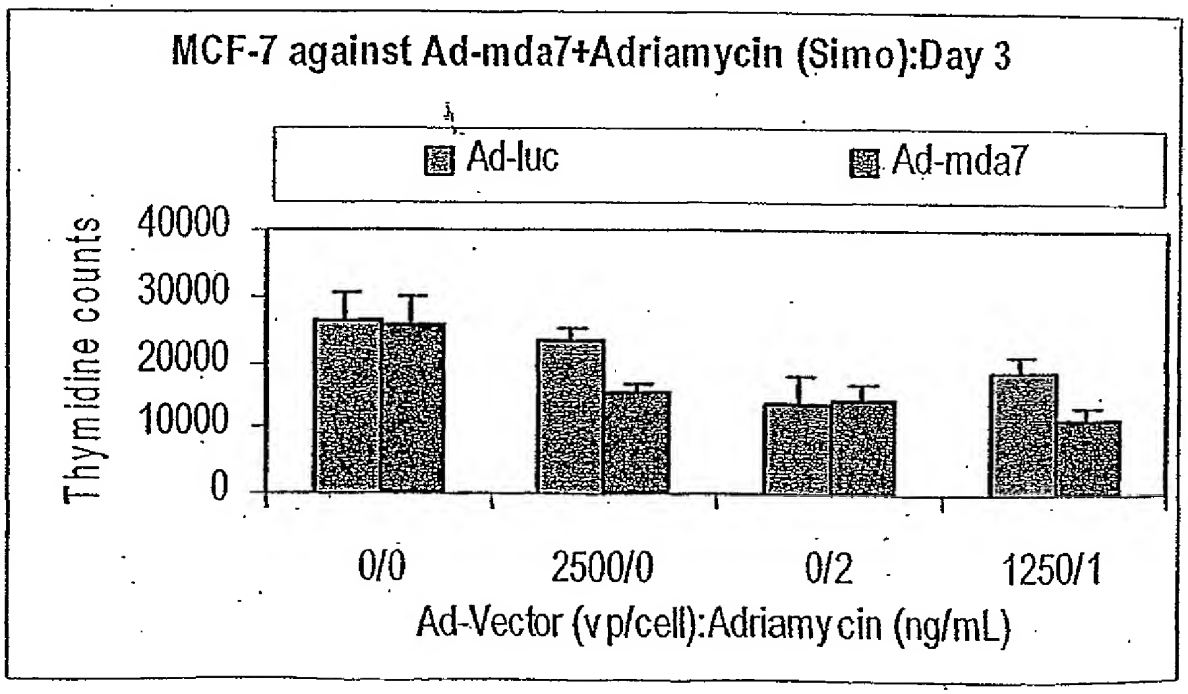
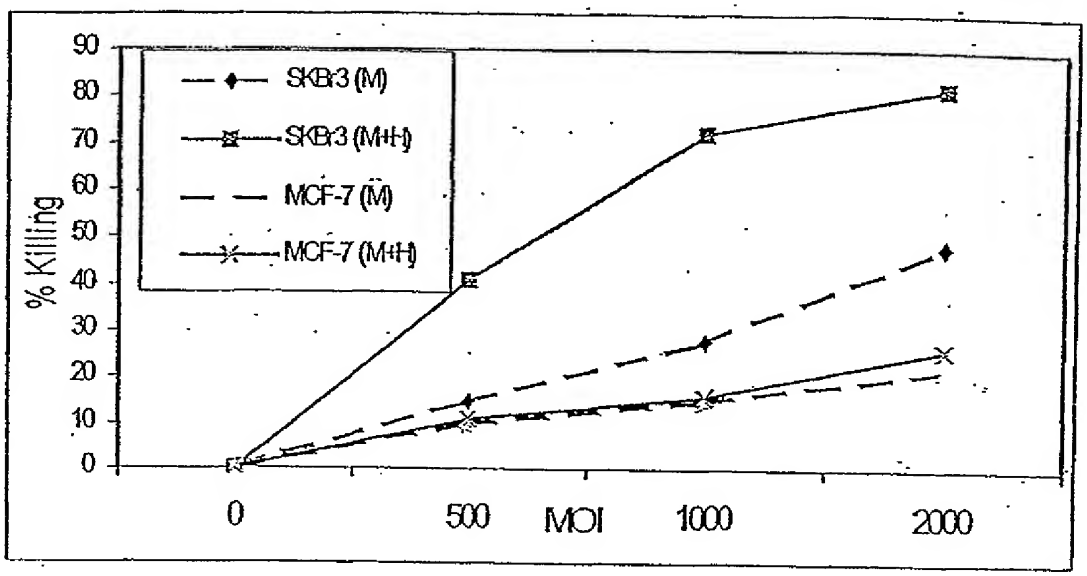



FIG. 13




| | |
|---|---|
| SKBr3 | Her2 + |
| MCF-7 | Her2 - |
| M | Ad-mda7 |
| H | Herceptin |

FIG. 15

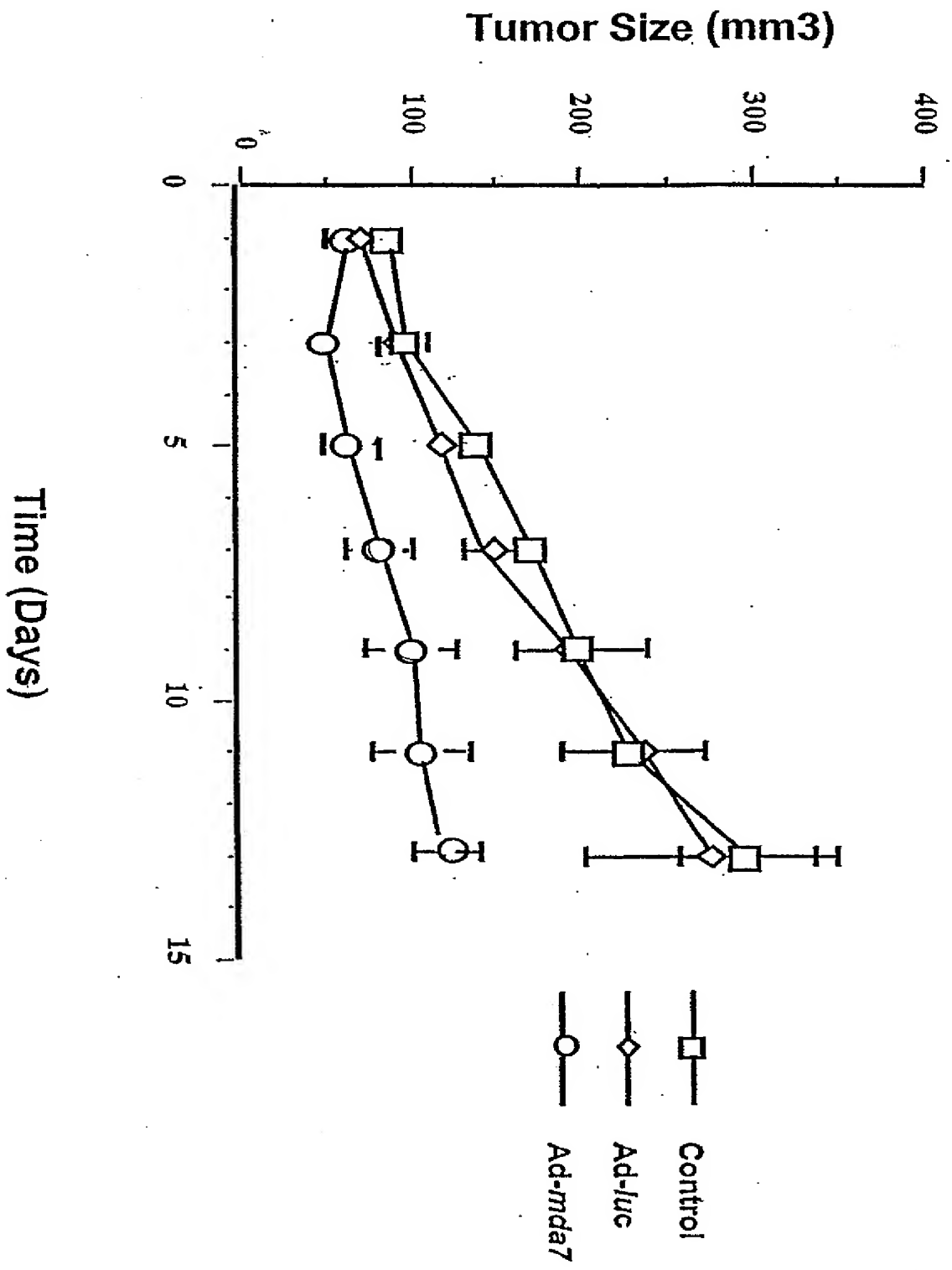

Tumor Size (mm3)
0
100
200
300
400
Time (Days)
0
5
10
15
Control
Ad-luc
Ad-mda7

FIG. 17

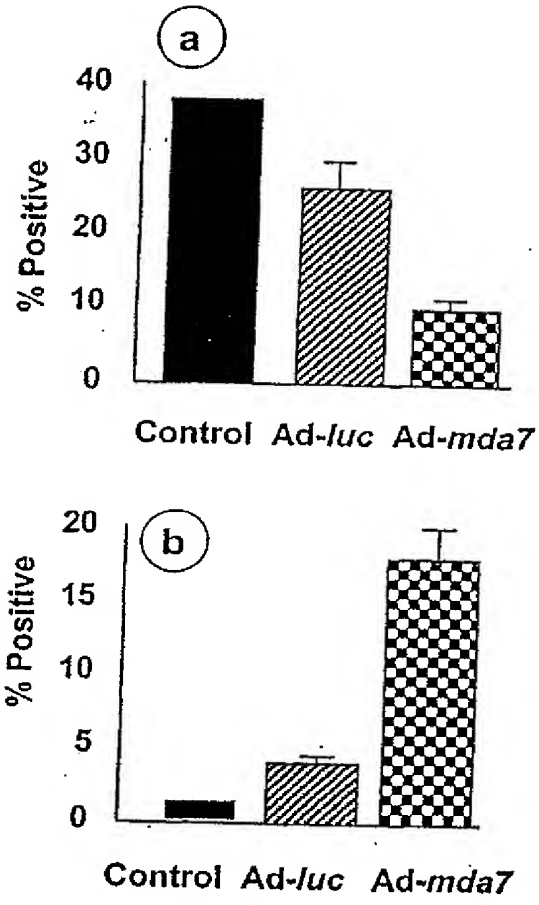

a
40
30
20
10
0
% Positive
Control
Ad-luc
Ad-mda7
b
20
15
10
5
0
% Positive
Control
Ad-luc
Ad-mda7

FIG. 19

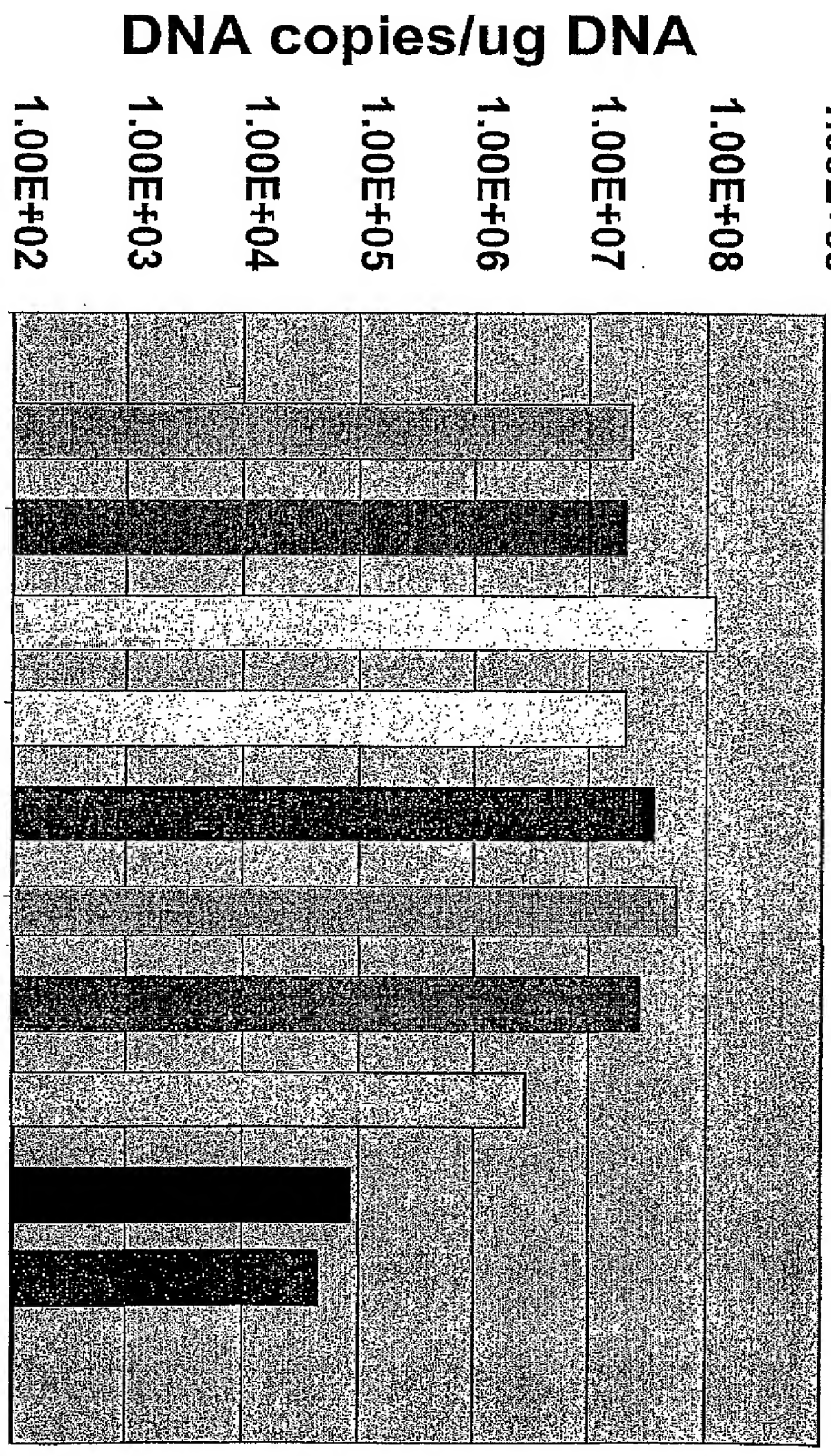
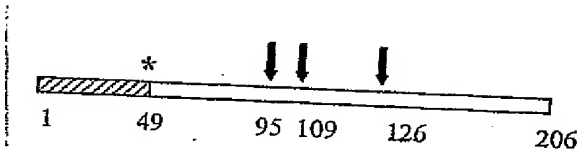

DNA copies/ug DNA
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
Center of Lesion
24 hrs
48 hrs
96 hrs

FIG. 21

A


*
1
49
95
109
126
206

B

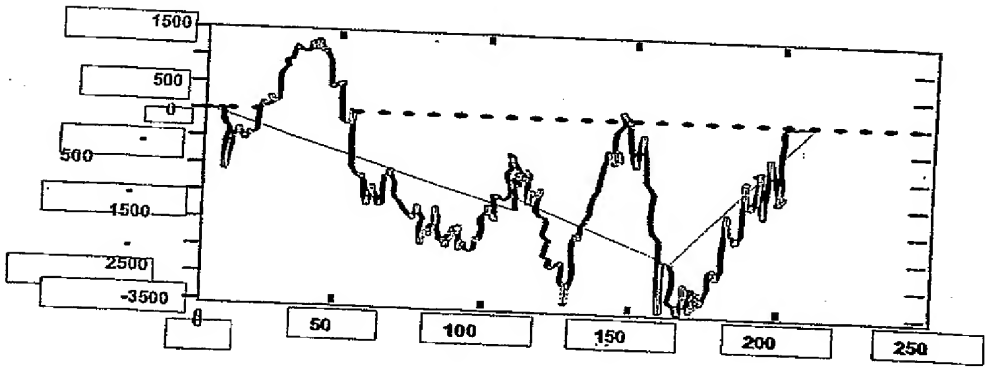

1500
500
0
500
1500
2500
-3500
0
50
100
150
200
250

FIG. 23

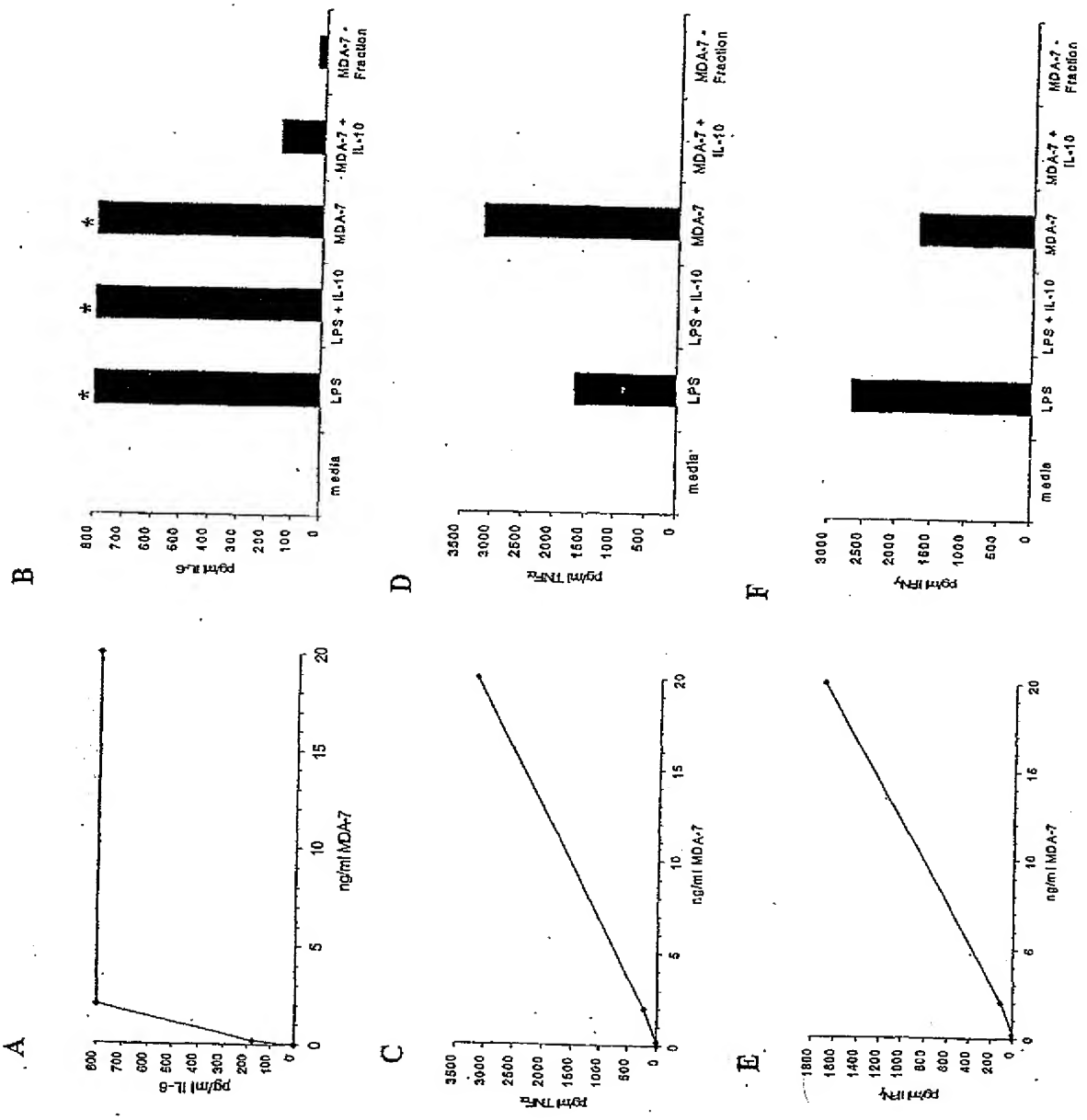

A
pg/ml IL-6
ng/ml MDA-7
B
pg/ml IL-6
media
LPS
LPS + IL-10
MDA-7
MDA-7 + IL-10
MDA-7 - Fraction
C
pg/ml TNFα
ng/ml MDA-7
D
pg/ml TNFα
E
pg/ml IFNγ
ng/ml MDA-7
F
pg/ml IFNγ

FIG. 25

```
Leu Pro Cys Leu Gly Phe Thr Leu Leu Leu Trp Ser Gln Val Ser Gly
          35                  40                  45

Ala Gln Gly Gln Glu Phe His Phe Gly Pro Cys Gln Val Lys Gly Val
      50                  55                  60

Val Pro Gln Lys Leu Trp Glu Ala Phe Trp Ala Val Lys Asp Thr Met
 65                  70                  75                  80

Gln Ala Gln Asp Asn Ile Thr Ser Ala Arg Leu Leu Gln Gln Glu Val
                 85                  90                  95

Leu Gln Asn Val Ser Asp Ala Glu Ser Cys Tyr Leu Val His Thr Leu
            100                 105                 110

Leu Glu Phe Tyr Leu Lys Thr Val Phe Lys Asn Tyr His Asn Arg Thr
        115                 120                 125

Val Glu Val Arg Thr Leu Lys Ser Phe Ser Thr Leu Ala Asn Asn Phe
    130                 135                 140

Val Leu Ile Val Ser Gln Leu Gln Pro Ser Gln Glu Asn Glu Met Phe
145                 150                 155                 160

Ser Ile Arg Asp Ser Ala His Arg Arg Phe Leu Leu Phe Arg Arg Ala
                165                 170                 175

Phe Lys Gln Leu Asp Val Glu Ala Ala Leu Thr Lys Ala Leu Gly Glu
            180                 185                 190

Val Asp Ile Leu Leu Thr Trp Met Gln Lys Phe Tyr Lys Leu
        195                 200                 205
```

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PCT

(10) International Publication Number
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(21) International Application Number: PCT/US2001/047215

(22) International Filing Date: 7 December 2001 (07.12.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
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(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
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Filed on 7 December 2000 (07.12.2000)

(71) Applicants *(for all designated States except US)*: **BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM** [US/US]; 201 West 7th Street, Austin, TX 78701 (US). **INTROGEN THERAPEUTICS, INC.** [US/US]; Suite 1850, 301 Congress Avenue, Austin, TX 78701 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **CHADA, Sunil** [US/US]; 4007 Waterview Court, Missouri City, TX 77459 (US). **GRIMM, Elizabeth** [US/US]; 6516 Brompton Road, Houston, TX 77005 (US). **MHASHILKAR, Abner** [IN/US]; 12414 Tottenham Drive, Houston, TX 77031 (US). **RAMESH, Rajagopal** [IN/US]; 2027 Cypress Bend Lane, Sugar Land, TX 77478 (US).

(74) Agent: **SHISHIMA, Gina, N.**; Fulbright & Jaworski L.L.P., Suite 2400, 600 Congress Avenue, Austin, TX 78701 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *with international search report*

(88) Date of publication of the international search report:
8 January 2004

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: METHODS OF TREATMENT INVOLVING HUMAN MDA-7

(57) Abstract: The present invention relates to gene therapy methods for the treatment of human disease. More specifically, the invention is directed to methods for treating a subject with an angiogenesis-related disease. In one embodiment, an adenoviral expression construct comprising a nucleic acid encoding a human MDA-7 protein under the control of a promoter operable in eukaryotic cells, is administered to said patient with a angiogenesis-related disease. The present invention thus provides for treatment of angiogenesis-related disease by through expression of mda-7 and inhibition angiogenesis. Such diseases include cancer.

WO 2002/045737 A3

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | SU ZAO-ZHONG ET AL: "The cancer growth suppressor gene mda-7 selectively induces apoptosis in human breast cancer cells and inhibits tumor growth in nude mice" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 95, no. 24, 24 November 1998 (1998-11-24), pages 14400-14405, XP002151807 ISSN: 0027-8424 cited in the application page 14400, right-hand column, paragraph 3 page 14404, right-hand column, paragraph 1 page 14405, left-hand column, paragraph 2 --- | 1-43 |
| X | SAEKI T ET AL: "Tumor -suppressive effects by adenovirus-mediated mda - 7 gene transfer in non-small cell lung cancer cell in vitro." GENE THERAPY, (2000 DEC) 7 (23) 2051-7., XP008013863 cited in the application page 2051, left-hand column, paragraph 1 -right-hand column, paragraph 1 page 2053, right-hand column, paragraph 1 page 2055, right-hand column, paragraph 3 --- | 1-43 |
| X | MADIREDDI M T ET AL: "A NOVEL MELANOMA DIFFERENTIATION ASSOCIATED GENE WITH PROMISE FOR CANCER GENE THERAPY" CANCER GENE THERAPY, NORWALK, CT, US, vol. 465, 2000, pages 239-261, XP000943276 ISSN: 0929-1903 cited in the application page 240, paragraph 3 page 245, paragraph 1 -page 253, paragraph 1 page 257, paragraph 1 --- | 1-43 |
| X | JIANG HONGPING ET AL: "The melanoma differentiation associated gene mda-7 suppresses cancer cell growth" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 93, no. 17, 1996, pages 9160-9165, XP002151808 ISSN: 0027-8424 cited in the application page 9160, right-hand column, paragraph 2 page 9164, right-hand column, paragraph 2 --- -/-- | 1-43 |

# INTERNATIONAL SEARCH REPORT

In ɔnal application No.
PCT/US 01/47215

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-43 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-43

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Application No
PCT/US 01/47215

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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| | | JP 2000516618 T | 12-12-2000 |
| | | NZ 334034 A | 27-10-2000 |
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| | | WO 9806441 A1 | 19-02-1998 |